ISOLATION AND METABOLISM OF RADIOACTIVE RETINOL, RETINAL AND RETINOIC ACID BY THE RAT *IN VIVO*

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Substances possessing vitamin A[1] activity were distinguished from other "accessory factors" in 1922 after a series of careful investigations by McCollum, Davis, Osborne, and Mendel (2-5). Since the early work with butter fat and cod liver oil, substances with vitamin A activity have been found in many plants (carotenoids) and animal tissues (mostly retinol esters) (6). The ingestion of diets containing inadequate amounts of these substances produces characteristic deficiency symptoms (7-11) which include (a) defective dark adaptation and ultimately complete loss of the visual process, (b) keratinization of epithelial tissues, (c) nerve degeneration, (d) cessation of growth, and (e) failure of the reproductive processes.

All symptoms of vitamin A deficiency can be prevented or relieved by adequate intake of carotene, retinol, retinal, or retinol ester (12-13). Retinoic acid is equally as effective as retinol in growth promotion (14-16) and in preventing cornification of vaginal epithelial tissue (17), but is inactive in promoting normal reproduction (18), or in preventing visual malfunctions, where retinal has a specific metabolic function (19, 20). Since retinoic acid apparently is not

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[1] In accordance with the definitive rules for the nomenclature of vitamins which were approved by the Commission on the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry (1), the terms retinol, retinal, and retinoic acid are used in place of vitamin A alcohol, vitamin A aldehyde (retinene), and vitamin A acid, respectively. The term vitamin A is used to refer to the general properties of retinol, its derivatives, and its provitamins.

reduced to retinol or retinal *in vivo*, either the free acid or one of its metabolites must be biologically active in growth promotion and in the maintenance of epithelial tissue.

Metabolic interrelationships among β-carotene, retinol, retinal, and retinoic acid have been reviewed by several investigators (21-23). Dietary β-carotene is converted to retinol ester in the intestine. Ingested preformed retinol, which exists in foodstuffs largely in the esterified form, is apparently hydrolyzed in the intestinal lumen and re-esterified in the intestinal wall. After absorption, esterified retinol is transported via the blood stream to the liver where it is stored largely as the ester (23). The vitamin A content of fasting normal blood remains nearly constant and consists almost entirely of free retinol, bound most probably to a high density α_1-globulin (24). On the other hand, post absorptive blood contains an increased amount of vitamin A in the ester form, which is probably bound to a low density lipoprotein (25). Liver retinol ester apparently is hydrolyzed by a liver esterase (21) and released into the bloodstream as retinol when needed by the tissues.

Retinal, although not a common dietary component, is absorbed from the intestinal lumen, reduced and esterified in the intestinal mucosa, and transported to the liver where it is stored as retinol ester (26). Enzymes which carry out the reversible oxidation of retinol to retinal are found in several tissues (26-29). Since the equilibrium of this reaction strongly favors reduction of retinal to retinol (30), retinal appears in low concentrations in mammalian tissues; indeed outside the retina it has been found only in the liver (31).

Another factor reducing the amount of retinal in tissues is the presence of enzymes in liver, kidney, and the small intestine (31-36), which catalyze the oxidation of retinal to retinoic acid. This oxidative reaction is essentially irreversible (33). Retinol ester is not recovered *in vivo* even after the administration of large doses of retinoic acid (37-39).

Although enzymes which oxidize retinol and retinal to retinoic acid exist in tissues, retinoic acid has never been found in any tissue when animals were fed with normal loads of β-carotene or retinol ester. On the basis of limited spectral evidence, Koizumi *et al.* claimed that β-carotene was converted to retinoic acid in tissue homogenates (40). Dimitrovski also reported that retinoic acid was formed by the rat *in vivo* after the administration of 10 to 60 mg of retinal and *in vitro* by rat intestinal preparations (35). In this case as well, the characterization of retinoic acid was limited to spectral analysis of crude fractions obtained by the treatment of intestinal extracts by alumina chromatography.

Even after massive dosing with retinoic acid, free retinoic acid has not been readily detected in tissues (39, 41-43). Recently, Jurkowitz, using an improved acidic extraction procedure, found retinoic acid in human plasma up to 6 hrs. after the oral administration of 100-200 mg of retinoic acid (44). After oral administration of 3 mg of retinoic acid to chicks, Krishnamurthy *et al.* (37) found retinoic acid in the liver 6 hrs. later, but not 18 hrs. later. After daily administration of 50-100 μg of retinoic acid to vitamin A deficient rats over a three week period, no retinol, retinol ester, or retinoic acid could be detected in any tissue (37). The difficulty in finding retinoic

acid as a product of administered precursors (31, 36, 37, 45) has led to the speculation that free retinoic acid is not an obligatory intermediate in the formation of a biologically active metabolite from retinol, but rather may be converted to that metabolite by another route (18, 22, 37).

A number of products of retinol metabolism *in vivo* have been suggested as possible links to the biologically active form of vitamin A. Wolf (46, 47) found that 5% of the radioactivity of 14-C^{14}-retinol injected intraperitoneally into rats appeared in expired CO_2 within 24 hrs. Appreciable amounts of radioactivity also appeared in the urine, feces, carcass, liver, intestine, blood, kidney, skin, and eyes. To a large extent, the radioactivity was water soluble. Further examination of radioactive components of the urine revealed two compounds separable by paper chromatography. One was water and ether soluble (WES), while the other was water soluble but not soluble in ether (WS). On the basis of spot tests for functional groups, it was concluded that WES contained double bonds, hydroxyl group(s), and an aldehyde group. WS was crystallized as a dinitrophenylhydrazone derivative, seemed to be a hydroxylated ester with a non-conjugated keto group, and had the elementary composition, $C_{11}H_{14}O_4$.

Garbers found radioactivity in the urine as well as in the α_2-globulin fraction of serum after the administration of radioactive retinol (24). In contrast, retinol was associated with the α_1-globulin fraction of serum. Varandani (48) also reported water soluble metabolites in the urine, but not in the liver, after the administration of radioactive retinol. After the administration of radioactive retinoic acid to vitamin A deficient chicks, Krishamurthy (37) found

radioactivity in a water soluble fraction after ether and acid-ethanol extractions. This water soluble material was dialyzable but was not biologically active.

After the intraperitoneal injection of 3 mg doses of radioactive retinoic acid, Yagishita et al. (41) found radioactive metabolites in the liver and intestine of rats. After intestinal and liver extracts were hydrolyzed and separated by thin layer chromatography, two radioactive compounds were detected, both of which were reported to be free of retinoic acid. The first was biologically active, was more polar than retinoic acid, contained hydroxyl groups as indicated by infrared analysis, and had an absorption maximum at 252 mμ, suggesting the presence of three conjugated double bonds. The second component was slightly less polar than retinoic acid on thin layer chromatography and was biologically inactive.

Radioactive retinoic acid administered to rats by Zile and de Luca (43) was not recovered from tissues as free retinoic acid, but as a group of metabolites separable by silicic acid chromatography. One metabolite was biologically active, non-acidic, less polar than retinoic acid, and absorbed at 272 mμ. Apparently this radioactive metabolite, which was isolated from liver, is not identical with that reported by Yagishita.

Ninety minutes after perfusion of an isolated rat liver with radioactive retinol, Zachman found that 75% of the radioactivity was recovered in a water soluble, hexane insoluble fraction (49). Ten percent of the radioactivity in the perfusate was secreted via the bile duct.[2] When he looked more closely at bile from bile duct cannulated

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[2] R. D. Zachman, unpublished observation

rats, he found 20 to 40% of the injected radioactivity in the bile 12 hrs. after the injection of radioactive retinol or retinoic acid via the portal vein (50). The radioactivity in the bile was separated into four fractions by step-wise anion exchange chromatography. The first fraction, which was eluted with methanol and was therefore non-ionic, contained about 10% of the recoverable radioactivity, but not retinal, retinol, or retinol ester. The second fraction, which was eluted with 1% acetic acid in methanol, had 10-20% of the recovered radioactivity. Although free retinoic acid would be eluted in this fraction, it was not looked for. The remaining fractions, accounting for 70-80% of the recovered radioactivity, were not further examined. Since bile, which has previously been ignored as a source of retinol metabolites, contained such a large portion of the injected radioactivity, the further characterization of these fractions should yield useful information about the metabolism of retinol.

As indicated by the preceeding discussion, the metabolites of retinol reported by various investigators apparently neither fit into a unified scheme nor seem to be related to one another. Although some of these metabolites had biological activity, none were well characterized. Unfortunately, the methods employed in these studies were rather crude. Metabolites were separated and characterized on the basis of their partition between various solvents or their extractability with organic solvents under given conditions, and by reactivity in non-specific tests such as the Carr-Price reaction. In the few cases where column chromatography was used, all components were not clearly separated. Thin layer chromatography, which is a very rapid and efficient method, cannot be expected to separate retinol compounds in pure form from

complex lipid mixtures containing hundreds of compounds. The mass of many of these components greatly exceeds that of the investigated compound. Moreover, retinol derivatives separated by thin layer chromatography are subject to destruction and isomerization (51).

Gas chromatography, which since its inception in 1952 (52) has been a valuable tool in analysis, has not heretofore been used successfully for the separation of retinol, its derivatives, and its metabolites. In the only reported application of gas chromatography to retinol separation, retinol, retinyl acetate, and retinyl palmitate were dehydrated rapidly to anhydro retinol (53).

As a result of the inadequate isolation and characterization of retinol metabolites, little is known about the metabolic pathway of retinol beyond the reversible oxidation of retinol to retinal. Although retinoic acid or one of its metabolites must be the active form of vitamin A in growth and epithelial tissue maintenance, free retinoic acid has not been clearly demonstrated as a product of retinol or retinal metabolism in vivo. Hopefully, the determination of the metabolic pathways involving retinol, retinal, retinoic acid, and further oxidized metabolites will ultimately elucidate the functions of vitamin A in metabolism.

Specifically, the present investigation concerns: (a) the recovery and unambiguous identification of free retinoic acid in the white rat after moderate doses of retinol or retinoic acid were administered, and (b) the characterization of metabolites found in liver, intestine, and bile after the administration of moderate doses of retinol, retinal, and retinoic acid.

The investigation of these problems has depended to a large degree upon the development of the following techniques for the separation and isolation of retinol and its derivatives: (a) gas liquid chromatography, (b) anion exchange chromatography with a gradient elution procedure, and (c) adaptation of existing techniques in thin layer chromatography and silicic acid chromatography.

MATERIALS AND METHODS

Materials

Radioactive retinol derivatives

C^{14}-retinol and C^{14}-retinoic acid were generously supplied by Hoffman-LaRoche, Inc., Basel, Switzerland. The alcohol was further purified by chromatography on alumina and the acid was further purified by either ion exchange chromatography or by silicic acid chromatography. C^{14}-retinal was prepared by MnO_2 oxidation (54) of C^{14}-retinol and was isolated by alumina chromatography.

Non-radioactive retinol derivatives

Non-radioactive crystalline all-*trans* retinol, all-*trans* retinyl acetate, all-*trans* retinal, and all-*trans* retinoic acid were purchased from Distillation Products Industries, Rochester, New York. The crystalline 13-*cis* isomer of retinoic acid was kindly supplied by Hoffman-LaRoche, Inc., Basel, Switzerland. The physical properties (melting point and ultraviolet absorption spectra) of these compounds agreed very closely with reported values (51, 55). In addition, 94-100% of the methyl ester of each compound was eluted as a single peak from gas chromatographic columns. The compound collected from the gas chromatographic eluate possessed the spectrum of the pure injected material. Therefore, these substances were used without further purification.

Solvents for column chromatography

Reagent grade methanol and acetic acid were used without further purification for ion exchange chromatography. For silicic acid chromatography reagent grade absolute ethanol was used as purchased. Spectral grade hexane was prepared from Skellysolve B (Skelly Oil Company, Eldorado, Texas) by treatment with fuming sulfuric acid (30% SO_3). Approximately 1500 ml of Skellysolve B were shaken for one hour with 250 ml of fuming sulfuric acid. The organic phase was then neutralized with 10% KOH, dried with Na_2SO_4, and distilled over KOH pellets. Approximately 1000 ml were collected. The purified solvent had a boiling range of 66-69° C and an absorbance of 0.05 or less at 230 mμ when measured in a cuvette with a 1 cm light path against a water blank.

Methods

Metabolic studies on bile cannulated rats

Fasted male rats (Rolfsmeyer Farm, Madison, Wisconsin) weighing from 150 to 300 g were anesthetized and their bile ducts were cannulated.[3] Then a solution of 1 ml of the appropriate substrate suspended in Tween 80 was injected into the portal vein. After closing the incision, the animal was placed in a restraining cage and the bile was collected at desired intervals (50). At predetermined times, the animals were killed, various organs were homogenized in $CHCl_3$:methanol (2:1), and the homogenates were filtered. The filtrates were evaporated to dryness and taken up in methanol. Bile and urine samples were diluted with 4 vols. of methanol. Diluted bile and urine and

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[3]The author wishes to gratefully acknowledge the preparation of bile duct cannulated rats by Richard D. Zachman.

methanolic extracts of organs were separated by ion exchange chromatography or used for other analyses.

Ion exchange chromatography of tissues

Analytical and semi-preparative columns (1.9 cm i.d. x 6.5 cm) contained 200-400 mesh Bio-Rad AG2-X8 anion exchange resin in the acetate form (Bio-Rad Laboratories, Richmond, California). Preparative columns (5.0 cm i.d. x 7.5-10 cm) contained either the above resin or Bio-Rad AG1-X4. Components of bile were separated well by both resins, indicating that 8% cross-linking was not excessive. Samples were eluted by gradient elution with increasing concentrations of acetic acid in methanol, according to the equation: $C = 1 - e^{-v/v_o}$ (56) where C is the concentration of the contributing solution found in the mixing chamber at elution volumn v, and v_o is the volume of the mixing chamber. The elution system consisted of three chambers (Fig. 1): the wash chamber, the distributing chamber, and the mixing chamber. A solution of 50% acetic acid in methanol (100% acetic acid for the preparative column) from the contributing chamber flowed into a closed 500 ml mixing chamber which emptied into the chromatographic column through one side of a three-way Teflon valve. The other side of the three-way valve was connected to the wash chamber, which contained methanol for washing the column and for the initial elution of sample. The effluent from the column was collected in 10 ml fractions by a Technicon drop counting fraction collector (Technicon Chromatography, Corp., Chauncey, New York). All parts of the system which came in contact with acetic acid were constructed of Teflon, glass, or stainless steel.

After equilibrating the resin column with methanol, 1 to 25 ml of a bile solution or organ extract from one rat was added to the column.

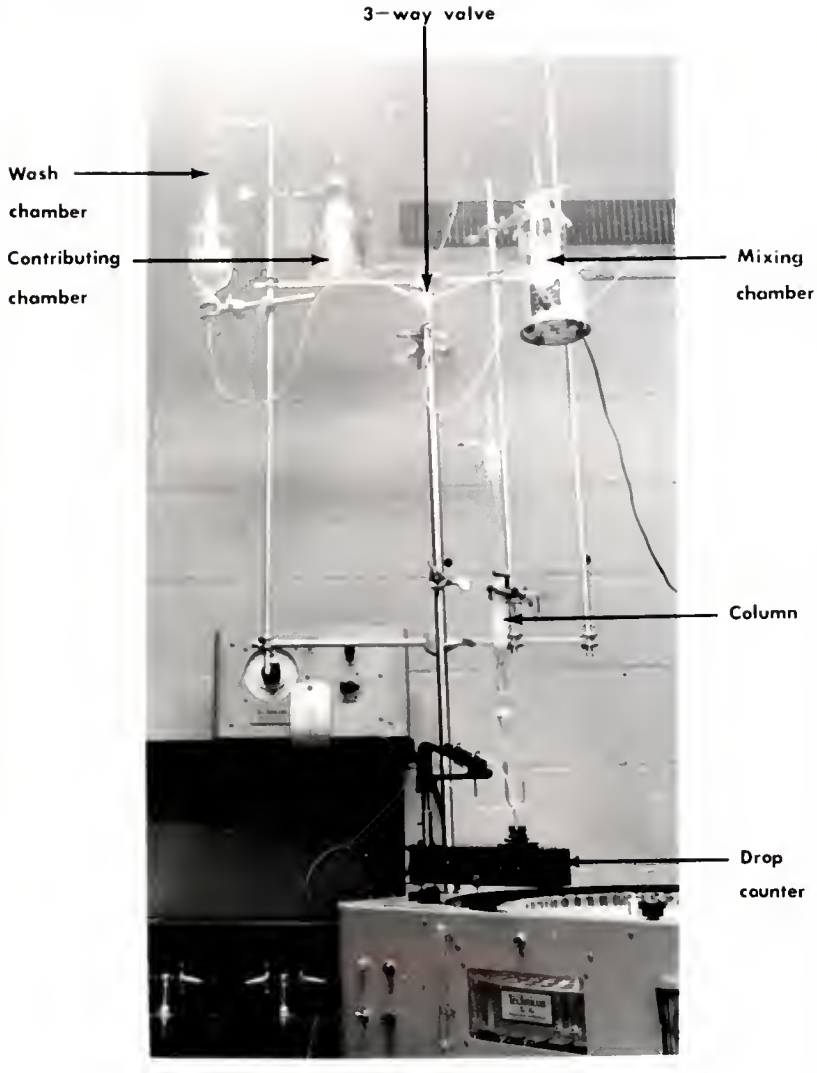

Fig. 1. Apparatus for automatic gradient elution chromatography. The solution from the contributing chamber passes into the mixing chamber, mixes with its contents and passes onto the column via the three-way valve. The column effluent is measured by the drop-counter and is collected in the fraction collector.

When the sample had completely entered the column, the column surfaces were washed twice with 2 to 5 ml of methanol. Thereafter 10 ml of methanol were added and the column was connected to the methanol reservoir. After 50 ml of methanol, which includes the sample and wash solutions, had flowed through the column into the fraction collector, the gradient was begun by switching the three-way valve to the mixing chamber. Eighty fractions were collected from the analytical columns and 150 from the preparative column. The column was regenerated by washing first with a 10% solution of sodium acetate until the eluate was basic; then it was equilibrated with methanol. Each tube was usually monitored for 350 mμ absorbing material with a Coleman Universal Spectrophotometer and was further analyzed for radioactivity. Radioactive fractions were frequently analyzed further by spectrophotometry or by other techniques as described in later sections.

Silicic acid chromatography of fraction III of bile[4]

Silicic acid chromatography was performed with the same apparatus as that used for ion exchange chromatography. The sample was separated on <300 mesh silicic acid which had been treated by the method of Hirsch and Ahrens (57) and was suitable for immediate use (Bio-Rad Laboratories, Richmond, California). The mixing chamber contained 500 ml of hexane, and the contributing chamber contained 25% hexane in absolute ethanol. Prior to sample separation, 20 g of silicic acid was poured into the column (1.9 cm i.d.) which was filled with hexane. After settling of the silicic acid, the sample was applied as described for ion exchange chromatography. A volume of 15 to 20 ml of hexane was run through the

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[4]The author wishes to thank E. Harris Meadows, Jr. for his excellent assistance in the chromatographic analyses performed on silicic acid columns and thin layer plates.

column before beginning the gradient. Fractions of 5 ml were collected in the automatic fraction collector.

Synthesis of retinol derivatives

Anhydro retinol was prepared by the dehydration of retinol with ethanolic HCl (58, 59). The reaction was stopped by neutralization with base, the mixture was extracted with benzene, and the anhydro retinol extract was finally purified by chromatography on activated alumina. Anhydro retinol was eluted with 0.5% acetone in hexane. The ultraviolet spectrum of the product (maxima are 346, 365, and 388 mμ) agreed with that reported by Shantz, Cawley, and Norris (58).

Methyl retinyl ether was prepared from retinol by a modified Williamson's synthesis (60). Retinol was shaken with n-butyl lithium in dry benzene for 5 minutes. Then dimethyl sulfate in dry benzene was added. The products were washed with acid and then base, dried over Na_2SO_4, and separated on activated alumina. Retinol ether, which was eluted with 1% acetone in hexane, was well separated from retinol and was used without further purification.

Methyl retinoate was prepared by treating retinoic acid with diazomethane in ether. The diazomethane was released from "Diazald" (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin) by treatment with NaOH and was distilled into ether (61). Diazomethane prepared in this manner was preserved for 2 or 3 days in the cold. Diazomethane was generated and used in a hood because of its poisonous and explosive nature. After evaporation of ethyl ether in vacuo, methyl retinoate was crystallized twice from methanol:H_2O (5:1). Its melting point and infrared and ultraviolet spectra agreed with those reported in the literature (62, 63).

Gas liquid chromatography (GLC)

A Model 600 Research Specialties Co. gas chromatograph was used for all gas chromatography. Solutions containing pure anhydro retinol, methyl retinyl ether, retinal, methyl retinoate, retinol, or retinyl acetate were used directly.

Some tissue extracts were analyzed without treatment, but most were methylated by addition of ethereal diazomethane until the evolution of nitrogen gas ceased. The resulting mixture was evaporated to dryness under N_2, and was dissolved in an appropriate amount of hexane, isopropyl ether, or other volatile solvent. Hydroxylated compounds were converted to the more volatile trimethyl silyl ethers by treatment with hexamethyldisilazane and trimethylchlorosilane in pyridine (64).

For solutions of pure compounds a capillary injection system gave reproducible results with 0.5 to 2.0 µl of solutions, but not with larger volumes. For tissue extracts an on-column injection system was used. Samples were delivered from a needle tipped Hamilton microsyringe through a rubber septum into the carrier gas stream. With this procedure larger more accurately measured samples (5-50 µl) could be analyzed with better reproducibility than with the capillary injection system.

Volatilized components of the sample were separated on 2 to 4.5 ft. columns containing a silanized diatomaceous earth support coated with 1 to 3% SE-30 (a non-polar silicone rubber gum made by General Electric Corp.) unless otherwise stated. Columns were treated with β-carotene to prevent the dehydration of retinal and retinyl acetate. From 50-300 µg of β-carotene in benzene were added to the column by either injection system. After raising the column temperature to 250° C for 2-3 hours

and then cooling to normal operating temperature, unstable retinol derivatives could be chromatographed with little destruction.

Components of the column effluent were detected in the gas phase by an argon ionization detector or, alternatively, by a flame ionization detector in parallel with an electron capture detector.

Individual components from the effluent stream were collected when desired by one of four methods. (a) The conventional collecting device was a glass U tube, shortened and tapered at the distal end, which contained a 4 mm layer of Al_2O_3 over a glass wool plug. Components were adsorbed onto the alumina and eluted with a minimum of 0.5 ml of absolute ethanol. When the collection device was properly made, essentially all the liquid and solid components of the effluent stream were adsorbed on the alumina. The alumina was frequently changed to prevent contamination from previously collected samples. (b) A rapid, inexpensive, and disposable collection system with which the collected sample could be eluted with less than 0.1 ml of most solvents was made from a 1.5 mm o.d. x 300 mm glass capillary tube. The capillary was connected to the 1/16 in. exit tubing from the detector by means of a #15 AWG Teflon tube, and could be rapidly inserted and removed. (c) Unstable compounds such as methyl retinyl ether, retinol, and retinyl acetate were collected on a cotton pad placed in the bottom of a hypodermic needle which was inverted over a short Teflon tube attached directly to the exit end of the column. Although collection was inefficient, unstable compounds which were destroyed in the long (75 cm) exit system could be isolated intact by this procedure. Retinol, retinyl acetate, and, to some extent, methyl retinyl ether were dehydrated to anhydro retinol when collected in the conventional manner.

(d) An automatic gas fraction collector (Packard Instrument Co.) was used to monitor the effluent stream for radioactivity. Sample components were condensed in anthracene filled cartridges and the entire cartridge was placed into a vial for counting in a liquid scintillation spectrometer. The efficiency of collection by this method was 75-85%, approximately the same as by adsorption on Al_2O_3 or by the capillary collection method.

Thin layer chromatography (TLC)

The apparatus was manufactured by Research Specialties Co., Richmond, California, and standard techniques were used. Samples were applied on TLC plates coated with Silica Gel G by means of 10 and 100 µl Hamilton syringes. After sample application, the TLC plates were developed with (a) the benzene, chloroform, methanol (4:1:1) system of Wolf (41), (b) H_2O, or (c) benzene, chloroform, methanol, acetic acid (5:5:5:1). Separated components were detected by fluorescence under ultraviolet light, by iodine vapor, or by charring in the presence of sulfuric acid. Detection by I_2 vapors proved to be the most sensitive method. Charring in the presence of H_2SO_4 did not reveal more spots than did I_2 vapor. A permanent record of the TLC chromatogram was conveniently made by photography with a polaroid 110 B Land Camera. Utilization of a blue filter (Corning No. 5-61 Signal Blue) between the plate and the lens gave a photograph with high contrast between the yellow I_2 spots and the white background of the plate. In many cases, spots which were invisible to the naked eye were revealed by this type of photography. The adsorbent was scraped off in suitable increments, suspended in scintillation fluid containing 4% Cab-O-Sil (65), and counted for radioactivity in a Tricarb liquid scintillation spectrophotometer.

Ultraviolet spectrophotometry

Most ultraviolet spectra were obtained on the Perkin-Elmer Model 4000 ultraviolet recording spectrophotometer. For compounds obtainable in sufficient quantity, standard 3 ml cells with 1 cm light paths were employed. However, the small quantities usually obtained from the effluent of the gas chromatograph necessitated the use of microcells containing 0.1 to 1 ml of solution. To adapt the recording spectrophotometer for this purpose, a blackened shield was placed into the light path of both the reference and sample beams. An orifice exactly 1 mm in diameter was drilled into each shield and the shields were adjusted to permit the light beams to pass through the center of the microcells. The intensity of the hydrogen lamp source was sufficient for the required analyses without beam condensation. The spectra of very dilute solutions obtained from ion exchange chromatography were measured in cells having 2 or 5 cm light paths. Two single beam spectrophotometers, the Beckman DU and the Zeiss PMQ II, were also used.

Methods for measurement of radioactivity

Radioactivity was measured either as infinitely thin dry samples plated on aluminum planchets in a windowless gas flow Geiger-Muller counter, or as a liquid suspension in scintillation fluid by means of a Tri-Carb liquid scintillation spectrometer. One to 10 ml of the radioactive solutions were diluted with either the dioxane-naphthalene scintillation fluid of Bray (66) or the toluene fluid recommended by Packard Instrument Co. (67). The former scintillation fluid was superior for counting water soluble compounds.

Fractions obtained from ion exchange chromatography were assayed for radioactivity by liquid scintillation spectrometry as aliquots of 0.1 ml to 10 ml. Since sequential fractions from an ion exchange column contained increasing amounts of acetic acid, which appreciably quenched the counting response, an internal standard (C^{14}-toluene) was necessarily added to each assay vial to determine the exact amount of radioactivity. Alternatively, the methanol-acetic acid eluant was evaporated from each fraction before addition of the scintillation fluid. Internal standards were always used in counting yellowish solutions, which showed significant quenching.

Specific activities of retinoic acid derivatives were calculated by dividing the radioactivity in a given solution by the concentration of retinoate derivatives in that solution. The concentration of retinoic acid was determined from the absorbance of the solution and the extinction coefficient of all-trans retinoic acid determined in this laboratory, namely an $E^{1\%}_{1\ cm}$ of 1470 at the wavelength of maximum absorption, which varied from 337 to 350 mμ depending on the solvent.

Hydrolysis of tissue extracts

Methanolic solutions of fraction I and III of liver and bile or of whole bile were treated with 0.1 to 0.2 ml of 50% NaOH and heated in a water bath at 60 to 70° under a nitrogen stream for 45 to 180 minutes. After neutralization of the hydrolysate with concentrated HCl and evaporation to dryness, the residue was suspended in 2 to 3 ml of ethanol and was again evaporated to dryness. The anhydrous residue was then extracted with methanol or ethanol and stored under nitrogen.

EXPERIMENTAL RESULTS

Analysis of Six Retinol Derivatives by Gas Chromatography

Gas chromatography of individual derivatives on short columns

By the use of short columns packed with an acid washed, base washed, and silanized support coated with 1% SE-30, anhydro retinol, methyl retinyl ether, retinal, and methyl retinoate chromatographed as virtually one substance without destruction (Fig. 2). For successful chromatography of methyl retinyl ether, the column was necessarily aged by passing carrier gas through it under normal operating conditions for one or two days prior to use. Retinol and retinyl acetate, on the other hand, dehydrated to form anhydro retinol even after prolonged aging of the column, in agreement with Ninomiya's report (53). These compounds could be chromatographed only by pretreating the column with an anti-oxidant (β-carotene) and increasing the carrier gas flow rate about six fold (Fig. 3). The treatment minimized dehydration of retinol and retinyl acetate for one to three days.

Identification of chromatographically separated compounds by ultra-violet spectra

In order to ascertain whether or not retinol derivatives were chemically changed by GLC treatment, the ultraviolet spectrum of each compound collected from the gas chromatographic effluent was compared with the spectrum of the corresponding original material. As shown in Fig. 4, the spectra of the collected and injected compounds agreed closely. The small amount of anhydro retinol present in chromatographed

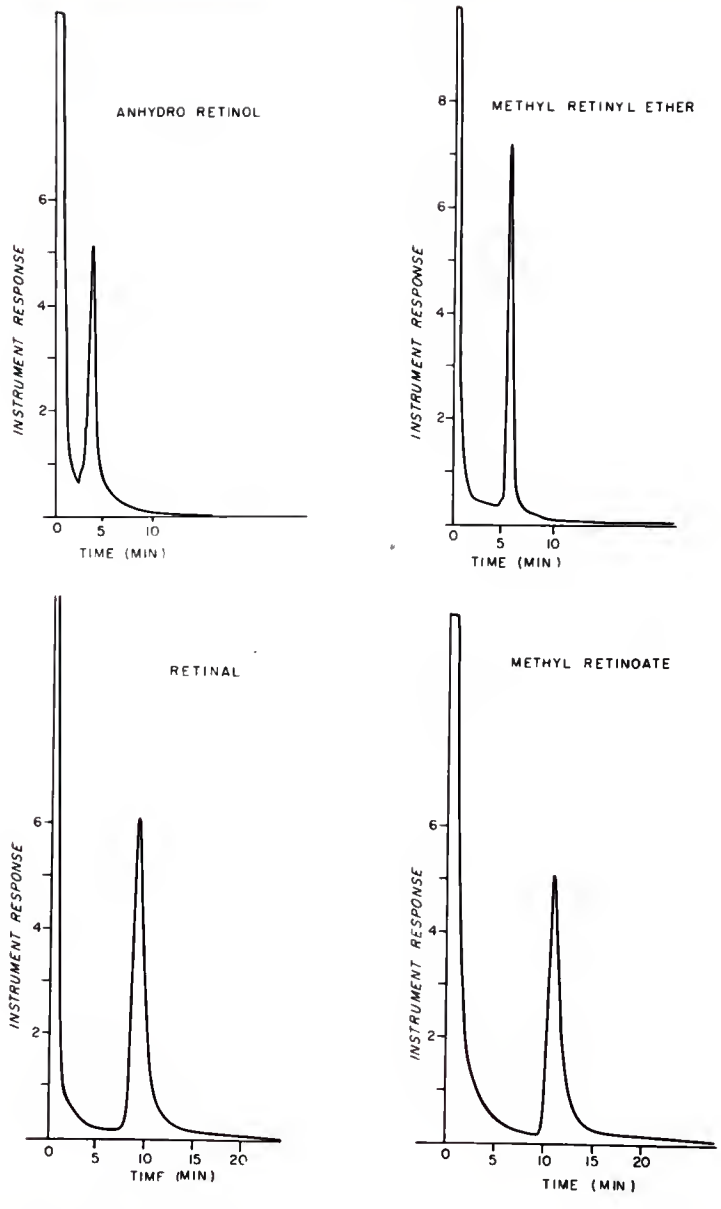

Fig. 2. Gas chromatography of individual retinol derivatives on Gas Chrom P. Approximately 1 μg of each derivative was chromatographed on a column of 60-80 mesh Gas Chrom P coated with 1% SE-30. Column length: 2 ft.; column temperature: 150° C; argon flow rate: 150 ml/min.

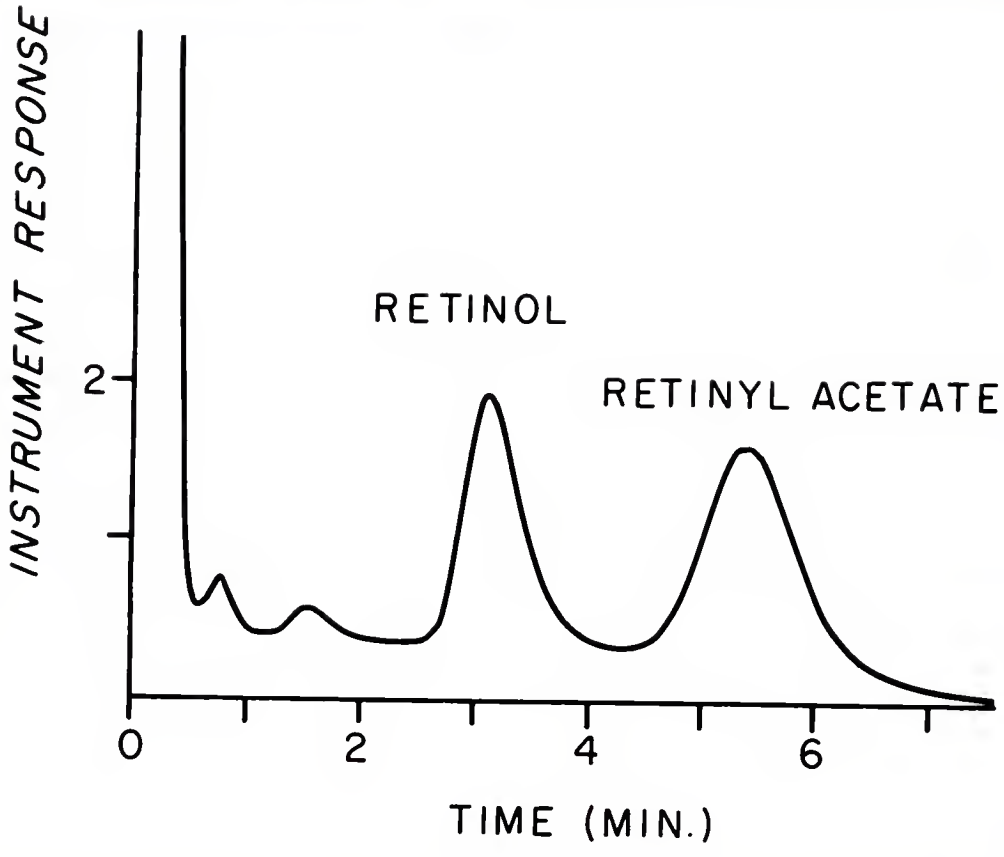

Fig. 3. Gas chromatography of retinol and retinyl acetate. Approximately 5 μg of each compound were separated on a column of Gas Chrom P coated with 1% SE-30 and treated with β-carotene as described in the text. Column length: 2 ft.; column temperature: 150° C; argon flow rate: 880 ml/min.

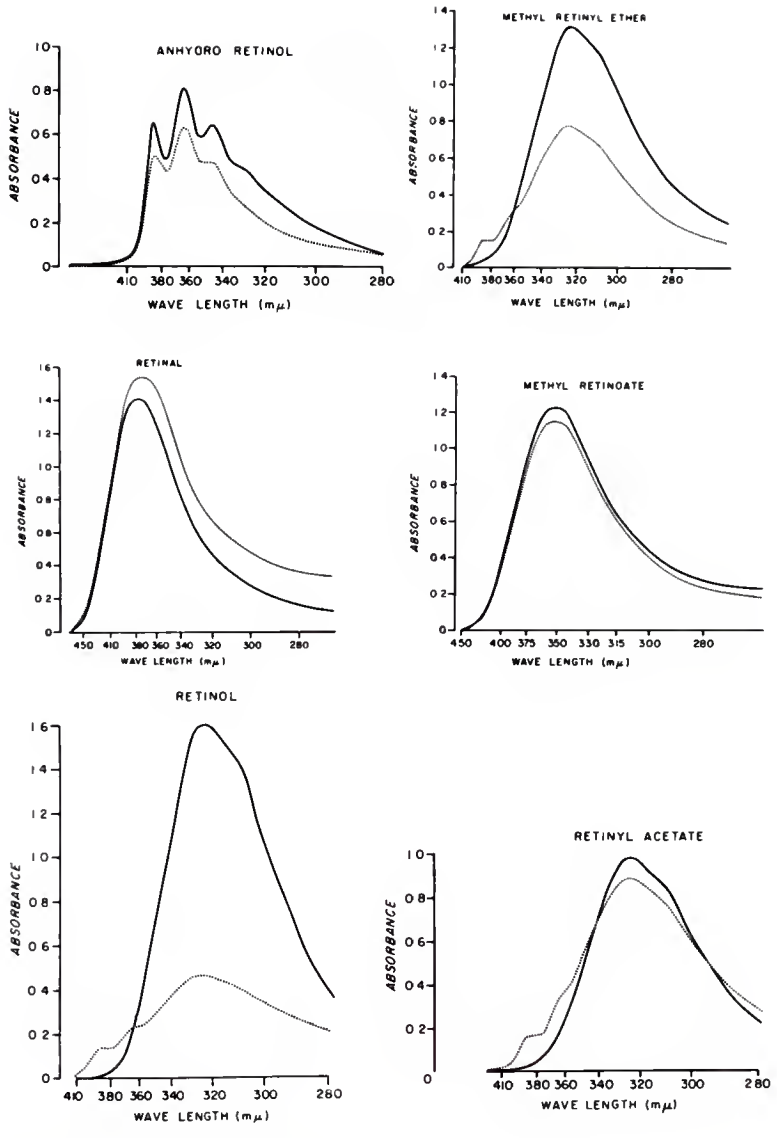

Fig. 4. Spectra of retinol derivatives before and after gas chromatography.

_____ Spectra of solutions applied to gas chromatography

..... Spectra of collected compounds

samples of retinol, retinyl acetate, and methyl retinyl ether apparently resulted from dehydration after separation on the column. When these compounds passed through the conventional detection system and were collected from the conventional output port, anhydro retinol accounted for 50 to 100% of each compound injected.

Except for retinol and retinyl acetate, the relative absorbance of the spectra shown in Fig. 4 indicate the amount recovered from the gas chromatograph. The compounds tested and their percent recoveries were anhydro retinol (78%), retinal (109%), methyl retinoate (94%), methyl retinyl ether (59%), retinol (<25%), and retinyl acetate (<25%). The low recoveries for the latter three compounds were caused by an inefficient collection system, and for the latter two, by the high flow rate and the high injection pressure as well. The above recovery data, although only approximate, do show that the more stable derivatives were chromatographed and collected without appreciable alteration.

The relationship between the amount injected and detector response

Although the recovery of injected material was complete only under special conditions, small amounts of anhydro retinol, methyl retinyl ether, retinal, and methyl retinoate could be quantitated with the capillary injection system. A mixture of these four compounds was separated sufficiently (68) to permit qualitative and quantitative analysis. Either peak height or peak area was proportional to the amount of these compounds injected down to 0.025 μg (Fig. 5). The slight deviation from linearity is apparently characteristic of the argon ionization detector, since the recovery of trapped material is linear with the concentration of the injected material (Fig. 6). The capillary injection system used for these experiments gave reproducible

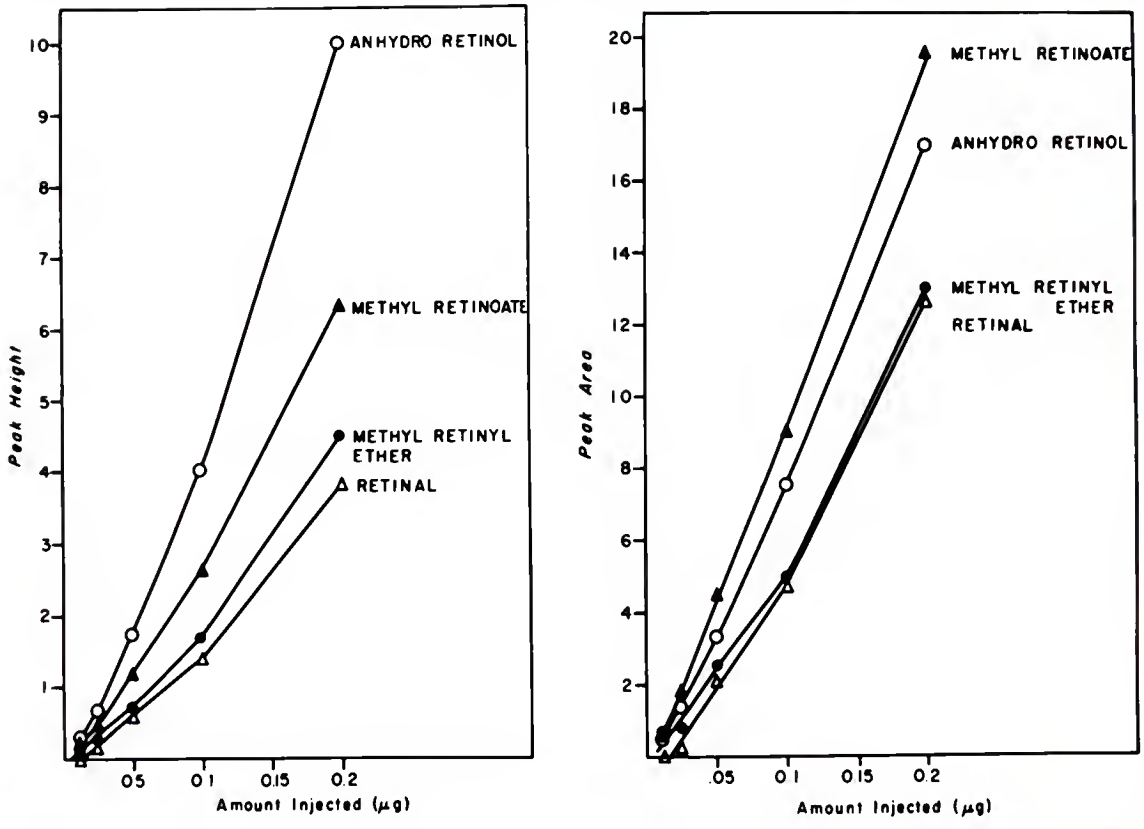

Fig. 5. Calibration curves for four retinol derivatives separated by gas chromatography. One microliter of solutions containing a mixture of the four derivatives shown above were separated at 150° on a 2 ft. column of Gas Chrom P coated with 1% SE-30.

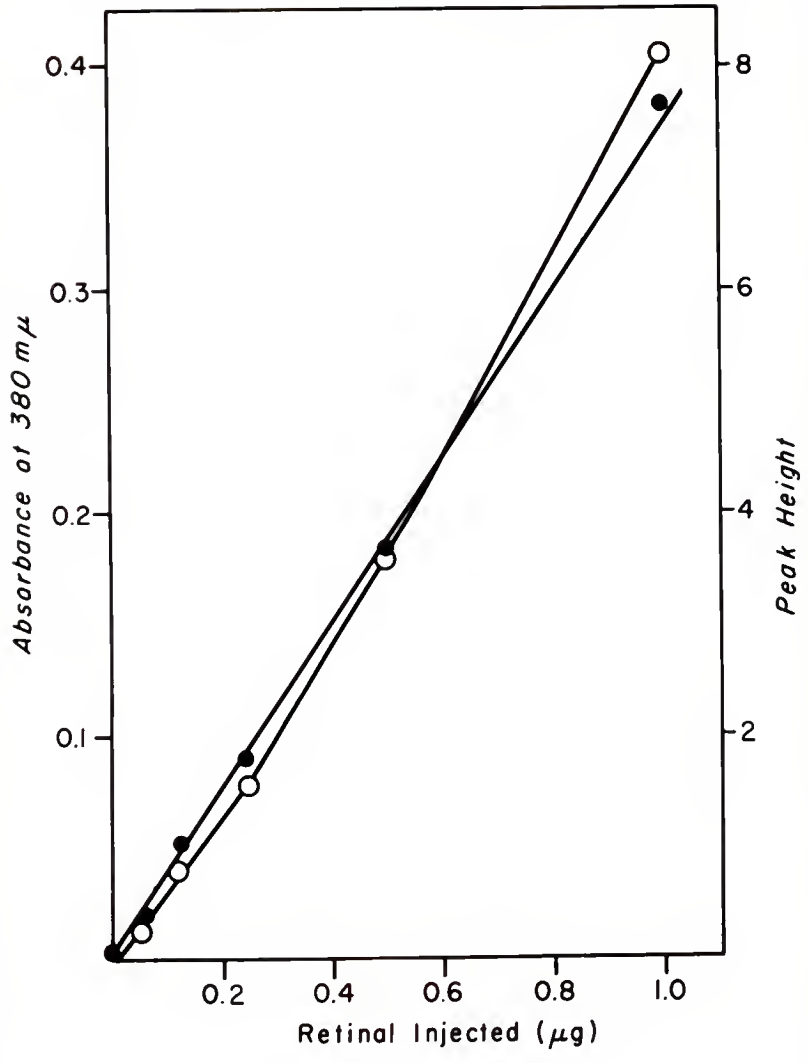

Fig. 6. Comparison of the recorder response with the amount of retinal recovered by gas chromatography. Retinal was chromatographed on a 2 ft. column of Gas Chrom P coated with 1% SE-30, collected on alumina at the output port, eluted with 0.5 ml of ethanol, and measured spectrophotometrically at 380 mμ. The heights of corresponding peaks on the recorder were directly measured.

—o—o— Peak height

—•—•— Absorbance of recovered retinal

results when the injection pressure was low or moderate but not when it was high. Hence, the dose-response relationship for retinol and retinyl acetate was not examined.

Electron capture detection of retinal and methyl retinoate

Certain types of molecules which have a high electron affinity, such as halogenated hydrocarbons, nitro compounds and conjugated carbonyls can be specifically detected by the electron capture method (69). Since retinal and methyl retinoate are conjugated carbonyls, their chromatographic behavior was monitored by splitting the column effluent through both flame ionization and electron capture detectors. Both detectors respond well to small amounts of retinal and methyl retinoate (Fig. 7). Since the electron capture detector responds poorly to the solvent, early components which are usually masked by the solvent response of the flame or argon ionization detectors can be measured by this device.

Effect of various factors on the stability of retinal and methyl retinoate during gas chromatography

Since retinal and methyl retinoate were stable under the initial mild conditions employed for short and medium columns (68), attempts were made to improve the resolution and efficiency of the column. A number of polar substrates were tried, such as QF1 (Dow-Corning), a fluoro-silicone polymer; XE-60 (General Electric Corp.), a cyano-silicone polymer; and ethylene glycol adipate (Applied Science Labs.), a high molecular weight polyester. Polar coatings inverted the elution order of retinal and methyl retinoate (compare Figs. 1 and 6) and increased the retention times relative to non-polar coatings.

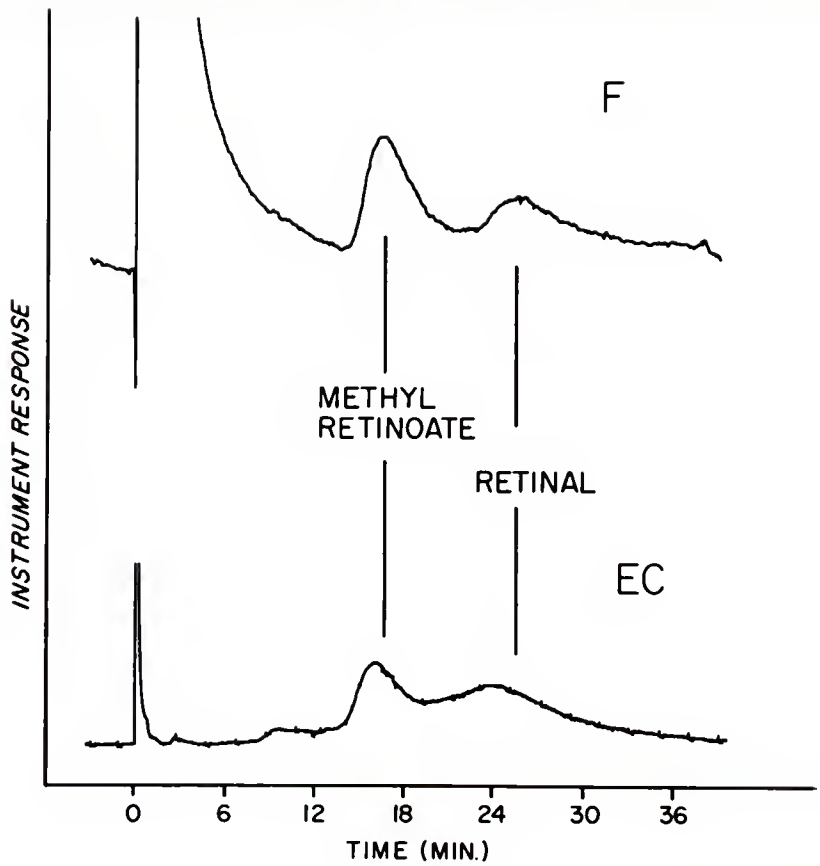

Fig. 7. Comparison of the responses of a flame ionization detector and an electron capture detector to a mixture of retinal and methyl retinoate. Approximately 0.2 μg of each component were separated by gas chromatography at 150° on a 2 ft. column of silanized Chromasorb W coated with 1% QF 1. The effluent stream was monitored in parallel with a flame ionization detector (F) and an electron capture detector (EC).

Three other observations should be mentioned: (a) Retinal and methyl retinoate were rapidly destroyed on silanized supports which were washed only with acid. Acid washed packing was only suitable for use after several days of aging, whereas a support neutralized with base after acid treatment was immediately useful. (b) The polyester type liquid phase contained an appreciable amount of alcohol and base soluble material which was probably unreacted adipic acid. Retinal and methyl retinoate were partially destroyed on this type of column, even after a considerable period of aging. (c) Exposure of the sample to uncoated adsorptive sites on the supporting medium seemed to increase the destruction of methyl retinoate. Some destruction of methyl retinoate occurred at 200° C on a 1% SE-30 coated Gas Chrom P column, while none was noticed at 230° C on a 3% SE-30 coated Gas Chrom P column with three times the length. In addition, adsorptive sites present in 1% coated supports increased the tailing of peaks, thereby reducing column efficiency. Ultimately the following chromatographic system was devised: liquid phase, 3% SE-30 on Gas Chrom P; column, 4.5 ft. glass; temperature, 180°; argon flow, 50-55 ml/min.; detector, argon ionization. The efficiency of this column was 1000 theoretical plates, four fold greater than that of earlier columns, and was suitable for the separation of methyl retinoate from tissue extracts.

Control Studies on Non-radioactive and Radioactive Retinoic Acid

Stability of retinoic acid to various conditions of analysis

Dilute solutions (5-10 μg) of retinoic acid were stored in the dark in non-polar solvents (hexane) at -20° for many months without any appreciable change or loss in optical density. A dilute solution of

retinoic acid in ethanol also showed no detectable loss of optical density or alteration of spectrum when stored in the cold under nitrogen for one week. On the other hand, when a similar solution was exposed to air and light in a Pyrex container for 5 hours, the optical density decreased about 4%/hr. A similar solution placed in a low actinic flask under air for the same period of time showed no detectable loss in optical density.

Crystalline retinoic acid as well as other retinol derivatives apparently can be kept for months or years when stored under nitrogen in the dark at -20° C. After a commercially available vial of retinoic acid was opened, the unused portion was placed in a low actinic bottle, stoppered with a serum cap, and then flushed with nitrogen through entrance and exit needles placed in the cap. Even after several months of storage, the crystals retained the same general appearance and melting point.

Accordingly, retinol derivatives in crystalline form and in solution as well as tissue extracts containing retinol derivatives have been handled in the dark and under N_2 as much as practicable, and have been stored in the cold. Since tissue extracts were occasionally subjected to light for considerable periods and fractions from ion exchange chromatography frequently remained in cold solutions of acetic acid and methanol for many days, a significant amount of isomerization or destruction of retinoic acid probably occurred.

Irradiation and isomerization of retinoic acid

When retinoic acid was applied to a TLC plate and left in air under subdued light for 30 minutes, several distinct zones representing materials more polar than retinoic acid appeared upon subsequent

development in benzene:$CHCl_3$:methanol (4:1:1). These zones were not present in retinoic acid chromatograms developed immediately after spotting. Since application of tissue extracts to a single plate frequently required 30-45 minutes, small amounts of retinoic acid in these extracts might be entirely transformed to more polar components during application. Thus the failure of Yagishita et al. (41) to find small amounts of retinoic acid in tissue extracts when analyzed by this procedure is understandable.

Upon ultraviolet irradiation of all-trans retinoic acid in ethanol (1 mg/ml) in a closed tube for 12 hours, extensive isomerization and some destruction occurred. On TLC analysis a new zone appeared which migrated faster than the all-trans isomer. This fast migrating zone fluoresced under UV light and probably is composed of cis isomers (51).

Three major components were separated when the methyl esters of the isomerate were analyzed by gas chromatography (Fig. 8). The relative retention times and relative amounts of each of these components, which are designated components (or peaks) A, B, and C, in the isomerate and in preparations of all-trans and 13-cis methyl retinoate are shown in Table I. The ultraviolet absorption spectra of all the collected components after gas chromatography of the methyl esters of the isomerate, the all-trans, and the 13-cis compounds were typical of methyl retinoate (see Fig. 4) and differed only in the wavelength of maximum absorption (from 348 to 359 mμ). Component A, which was found only in the isomerate, had an absorption maximum of 348 mμ. Component B, also found in smaller amounts as an impurity in preparations of the all-trans and 13-cis isomers, had an absorption maximum of 349 mμ.

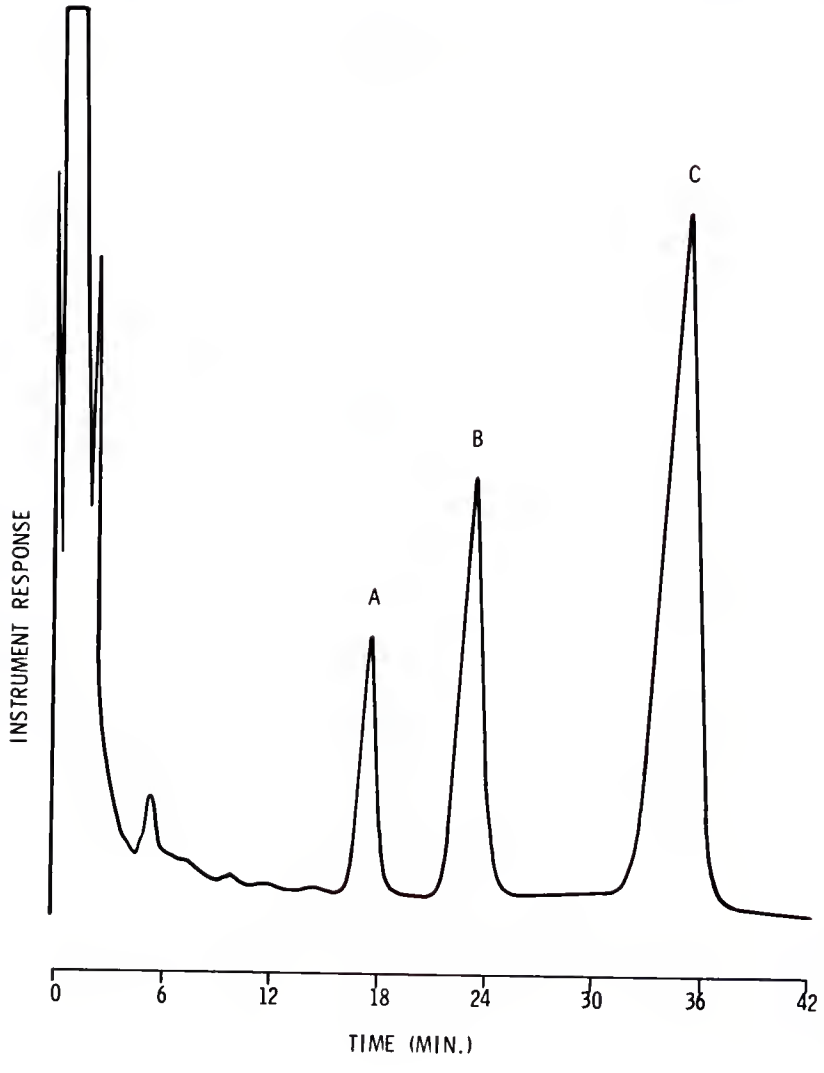

Fig. 8. Gas chromatography of the methyl esters of an isomerate of retinoic acid. Preparation of the isomers is described in the text. Separation was performed on a 4.5 ft. column of 100-120 mesh Gas Chrom P coated with 3% SE-30. Column temperature: 180° C; argon flow rate: 55 ml/min.

Table I

Comparison of isomers of methyl retinoate

Sample	Retention time	Absorption maximum of collected methyl ester	Reported maximum	% of total peak area
	minutes	mμ	mμ	
Isomerate produced from ultraviolet irradiation of all-*trans* retinoate				
Peak A	7.65	348	---	12
Peak B	10.10	349	---	25
Peak C	15.00	355.5	---	63
All-*trans* retinoate				
Peak B	10.15	349	---	4
Peak C	15.00	355.5	354	96
13-*Cis*				
Peak B	10.15	---	---	4
Peak C	14.80	358.5	359	96

Since several cis isomers have this same absorption maximum (62), absolute identification was not possible from spectra alone. Component C of the isomerate probably consists mainly of all-trans retinoate, since both its retention time and absorption maximum (355-356 mμ) agree with those of standard all-trans retinoate. Since the major peak of the 13-cis isomer also coincided with component C, however, this component of the isomerate may also contain one and perhaps more cis isomers.

Since the absorption maxima of the methyl esters of the all-trans and 13-cis isomers are unchanged by GLC analysis, isomerization of these isomers was apparently minimal during gas chromatography. This agreeable finding supports the above interpretation of multiple peaks in retinoate preparations, and might reasonably be extended to include the rapid separation of all six isomers of methyl retinoate.

Efficiency of methylation with diazomethane and recovery from gas chromatography

One milligram of all-trans retinoic acid was esterified with diazomethane in the manner described in the Methods section, and the resultant solution was compared spectrophotometrically with the initial retinoate solution. No significant change in absorbance occurred, although the absorption maximum shifted in the expected way for ester formation, from 348 mμ to 352 mμ. Upon gas chromatography of the esterified retinoic acid solution, components B and C accounted for 2% and 76% of the injected sample, respectively. Under similar conditions, pure crystalline methyl retinoate gave isomers B and C in yields of 2% and 79% of the injected dose, respectively. Thus diazomethane completely esterifies retinoic acid without destruction or isomerization. The 80% yields observed in these experiments were typical of a system consisting of top-of-column injection and capillary collection.

Analysis of purified radioactive retinoic acid

Since the 6,7-C^{14}-retinoic acid (reported specific activity, 43,800 $\frac{dpm}{\mu g}$) used in these experiments had been stored in a benzene solution for about 4 years prior to usage, it was purified before administration to test animals. When purified by anion exchange chromatography, most of the radioactivity appeared in fraction II, where non-radioactive retinoic acid is also eluted. Fraction II was extracted with ether, the ether was evaporated, and the residue was dissolved in ethanol to yield a stock solution of purified undiluted radioactive retinoic acid. Upon rechromatography of this purified undiluted radioactive retinoic acid on an anion exchange column, 99.8% of the recovered radioactivity was again found in fraction II. Upon gas chromatography of the methyl ester of the purified undiluted labeled retinoic acid, three major radioactive components appeared (Fig. 9). These components corresponded exactly with those found in the isomerate of unlabeled retinoate (Fig. 8). The last two components, B and C, had the typical absorption spectrum of methyl retinoate with absorption maxima of 348 and 354 mμ, respectively. Component A was present in insufficient quantity for spectrophotometric analysis. Of the total radioactivity collected in the eluate from the gas chromatograph, 6% was found in component A, 10% in B, and 33% in C. The overall recovery was much lower than with diluted samples of labeled retinoate, probably as a result of the destruction or adsorption during GLC analysis of part of the small quantity (about 0.2 μg) of retinoic acid injected.

For metabolic studies, purified undiluted radioactive retinoic acid was diluted with pure non-radioactive all-trans retinoic acid to give a solution of labeled substrate retinoic acid with a specific

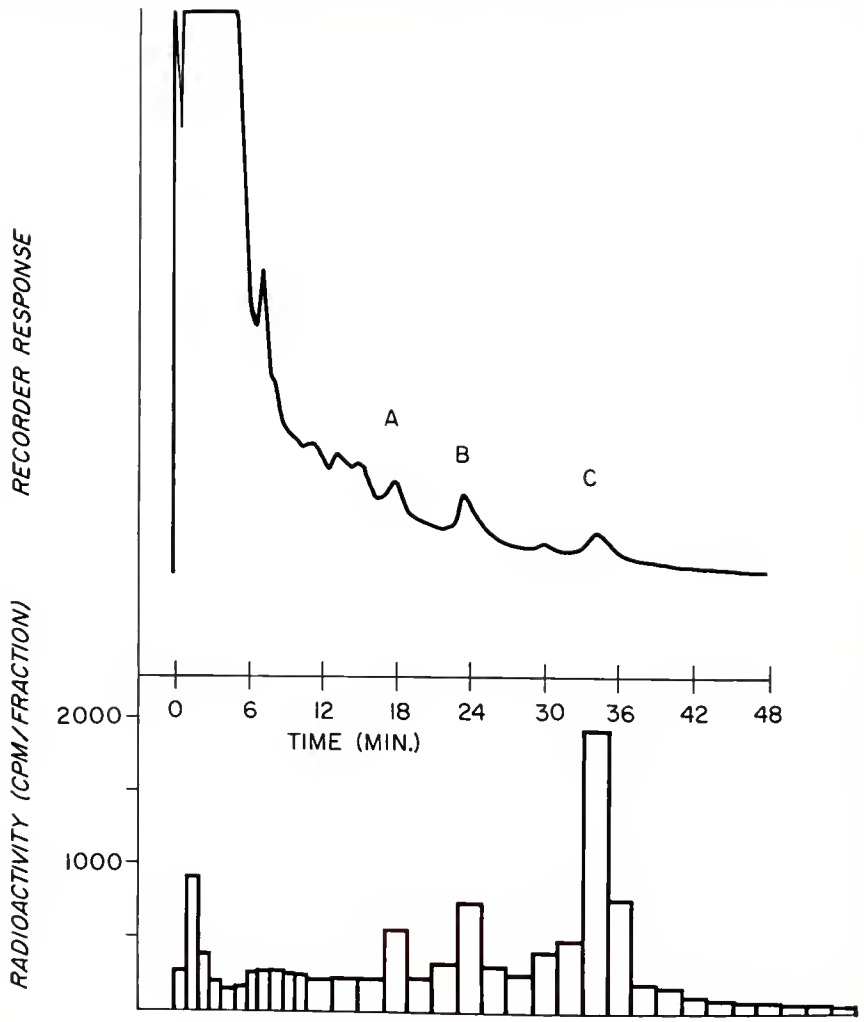

Fig. 9. Gas chromatography of radioactive retinoic acid purified by ion exchange chromatography. Twenty microliters containing approximately 0.2 μg of methylated radioactive retinoic acid were injected into the gas chromatograph and collected on anthracene crystals. Radioactivity was measured as described in the text. The upper trace is the response from the argon detector. The lower trace shows the corresponding radioactivity.

activity of 237 $\frac{dpm}{\mu g}$. Upon gas chromatography of this solution, component B contained 15% and component C, 67% of the total injected dose. When labeled substrate retinoic acid was recrystallized with 5 mg of non-radioactive all-*trans* retinoic acid, the specific activity after four crystallizations was 53% that of the original solution. This procedure measures only the radioactivity in the all-*trans* isomer. Thus, component C of the labeled substrate retinoic acid apparently contained 53% of the total radioactivity as all-*trans* retinoate and 14% as the 13-*cis* or as other *cis* isomers.

Undiluted radioactive retinoic acid (43,800 $\frac{dpm}{\mu g}$) was isomerized to a lesser degree when purified by silicic acid chromatography than by ion exchange chromatography. Radioactive retinoic acid was eluted from silicic acid with 5% acetone in hexane, methylated, and separated by gas chromatography (Fig. 10). In this case components A, B, and C contained 2%, 7%, and 45% of the recovered radioactivity, respectively. When undiluted radioactive retinoic acid purified by silicic acid chromatography was diluted with non-radioactive all-*trans* retinoic acid to a specific activity of 522 $\frac{dpm}{\mu g}$ and analyzed by gas chromatography, components B and C accounted for 7% and 60% of the recovered radioactivity, respectively. In both instances component C contained relatively more radioactivity and components A and B less than when ion exchange columns were used.

The presence of isomers of retinoic acid in the labeled substrate used for metabolic studies was both a help and a hindrance. The isolation of three isomeric components of retinoate from biological materials was useful as an additional criterion for identifying retinoate and its conjugates. On the other hand, the presence of isomers with

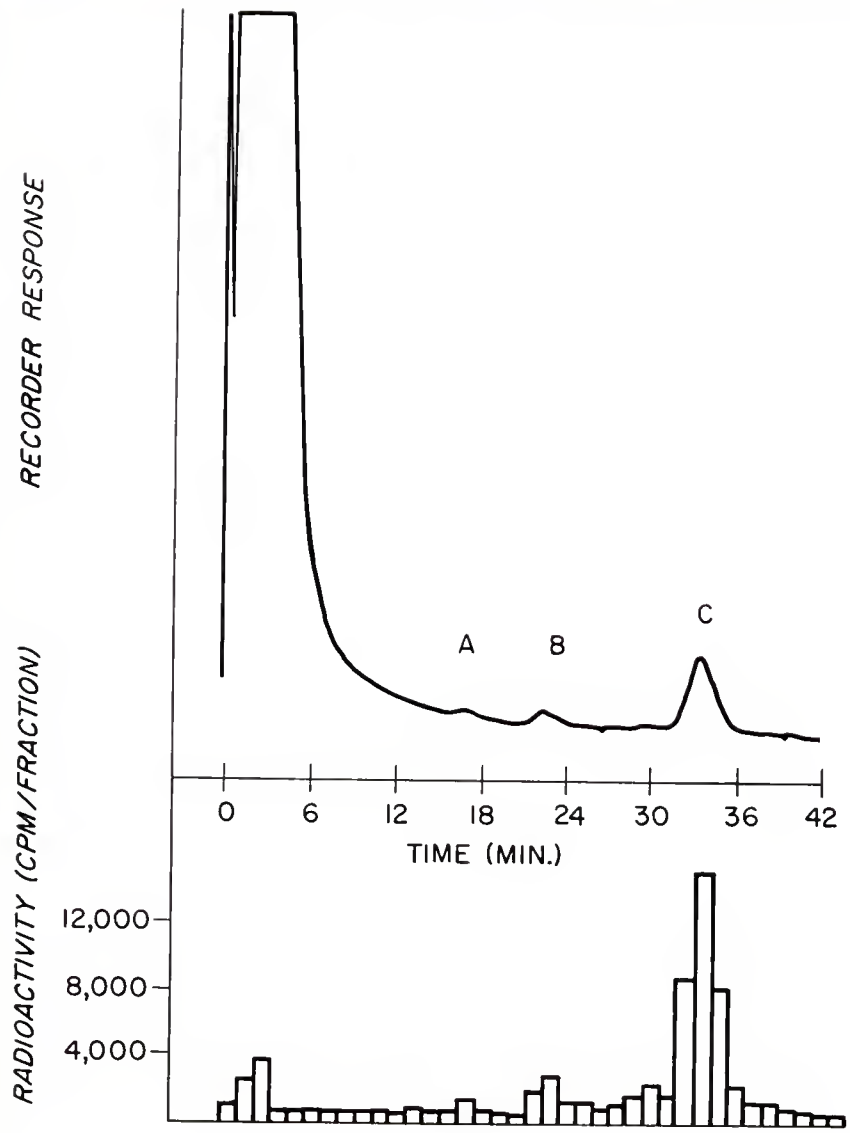

Fig. 10. Gas chromatography of radioactive retinoic acid purified by silicic acid chromatography. Radioactive retinoic acid was purified on a silicic acid column, methylated, separated by gas chromatography and collected on anthracene crystals. The upper trace is the argon detector response. The lower trace shows the corresponding radioactivity.

widely different specific activities in the injected substrate complicated the interpretation of radioactivity data in biological experiments. Since a major objective of this study was the elucidation of the metabolic pathway for retinoic acid, whatever its isomeric form, the existence of isomers in the substrate did not appreciably affect the experimental approach or the conclusions reached.

Distribution of Radioactivity in Various Tissues After the Administration of Labeled Substrates

Moderate doses of retinol, retinal, and retinoic acid were administered to bile duct cannulated rats in the manner described in the Methods section. Gradient elution ion exchange chromatography of extracts of liver and intestine, and of methanolic solutions of bile, resolved the radioactivity into three distinct fractions: fraction I contained non-ionic compounds, fraction II contained any free retinoic acid and other material with similar ionic properties, and fraction III contained more acidic compounds. The relative amount of radioactivity found in various tissues after the administration of each substrate is shown in Table II.

The bile became increasingly radioactive for the first 6 hours after the administration of 1-5 mg of retinol, retinal, or retinoic acid. The cumulative amount excreted in the bile in 6 hours was 16% with retinol, 28% with retinal, and 38% with retinoic acid (Fig. 11). The distribution of bile radioactivity on ion exchange chromatography was similar with all substrates (Fig. 12). Of the recovered radioactivity, approximately 10-15% was found in fraction I, 15-20% was found in fraction II, and 60-70% was found in fraction III.

Table II

Radioactivity recovered from various tissues after administration of 6,7-C^{14}-retinol derivatives

Substrate	Experiment	Dose		Time of organ analysis	Bile*	Liver	Intestine	Urine	Total recovery
		mg	cpm or dpm	hours	% of injected dose				
Retinol	2-24	1.3	10 $\times 10^6$ cpm	4	14				
				24	22	27	0.2	-	49
	2-12	2.5	6.3×10^4 cpm	4	10				
				24	17	31	-	-	48
	8-10	2.0	1.6×10^6 cpm	6	10	24	1.4	<0.1	36
Retinal	3-12a	2.8	3.4×10^6 dpm	5.5	18	29	-	0.2	44
	2-29a	3.1	4.4×10^5 cpm	6	34	22	0.7	-	53
	2-29b	3.1	4.4×10^5 cpm	4	31				
				24	37	36	0.4	-	73
Retinoic acid	2-3	2.2	2.0×10^5 dpm	2	13	11.3	-	-	24
	2-16	4.7	7.2×10^5 dpm	4.5	23	6.6	4.2	-	34
	4-5	3.8	9.4×10^5 dpm	7	45	5.3	1.4	-	52

*Cumulative amount

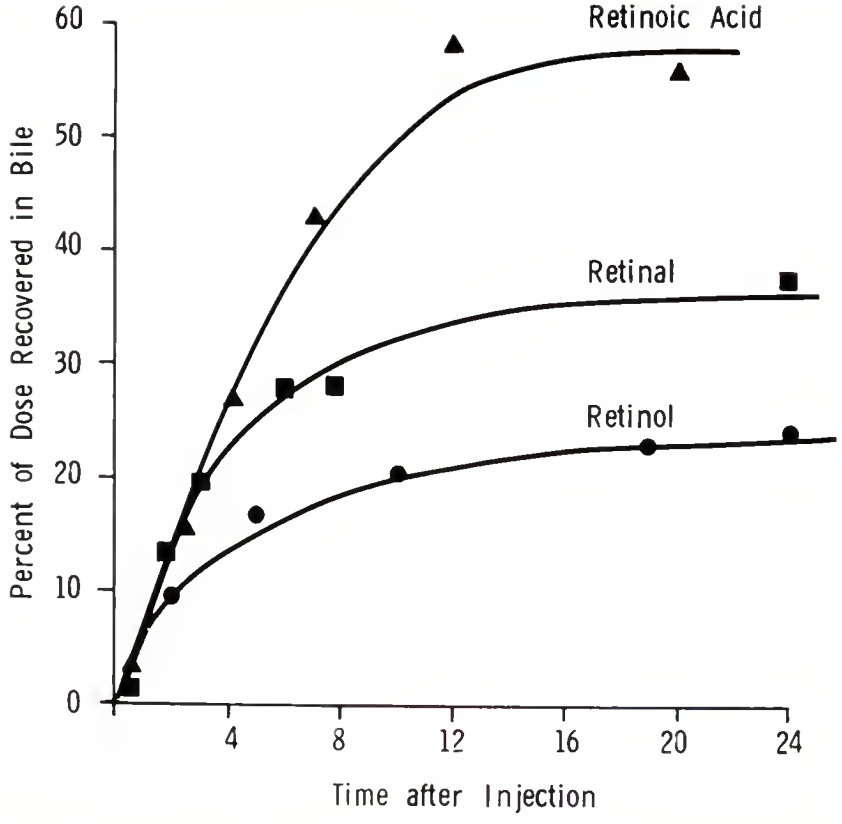

Fig. 11. The cumulative excretion rate of radioactivity into the bile after the administration of 1 to 3 mg of C^{14}-retinol, C^{14}-retinal, or C^{14}-retinoic acid. Plotted values represent averages of two, four, or twelve rats, respectively.

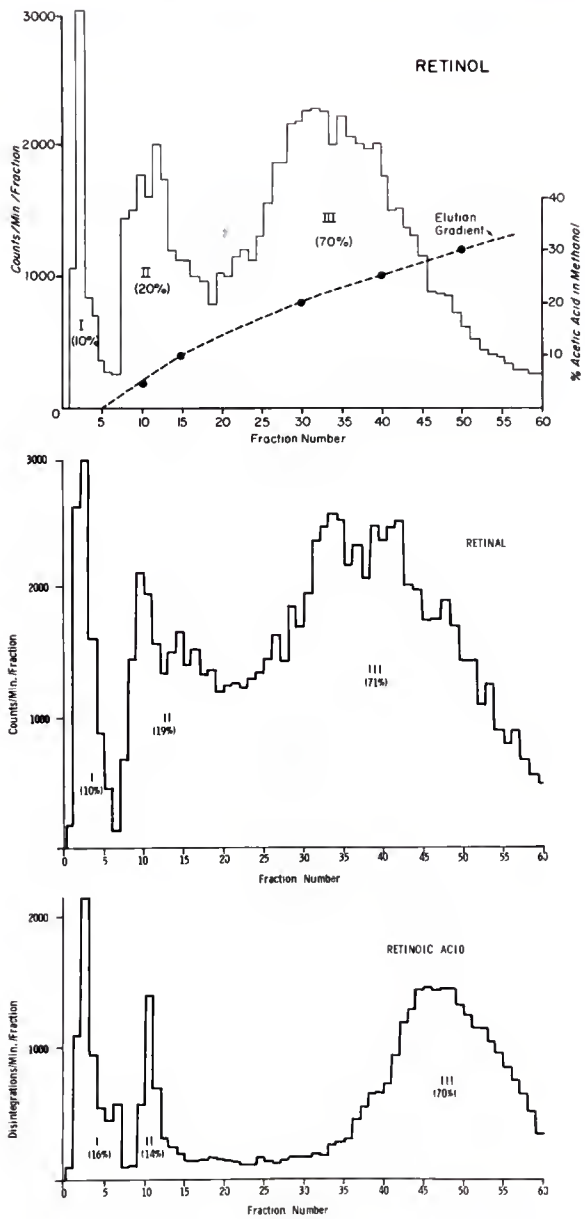

Fig. 12. Separation of bile by ion exchange chromatography after administration of C^{14}-retinol, C^{14}-retinal, or C^{14}-retinoic acid. Bile was collected for a 6 hr. period immediately following administration of each substrate, and was treated as described in the text prior to chromatography.

The liver contained approximately 30% of the injected dose within 6 hours after the intravenous injection of retinol or retinal, with 97% of the recovered radioactivity in fraction I (Fig. 13). Virtually all of the radioactivity in fraction I was found in the retinol ester fraction on alumina chromatography.[5] On the other hand, 4.5 hours after the injection of retinoic acid, only 7% of the injected dose was recovered in the liver, most of which appeared in fraction II where free retinoic acid is eluted.

The small intestine contained only 1% of the injected dose 6 hours after administration of retinol or retinal (Table II). Ion exchange chromatography showed that most of this radioactivity was in fraction I (Fig. 14). On the other hand, 4.5 hours after retinoic acid administration, about 4% of the injected radioactivity appeared in the small intestine, where it was distributed evenly between fractions II and III (Fig. 14).

Characterization of Free Retinoic Acid in Fraction II of Bile, Intestine, and Liver After the Intravenous Administration of Retinoic Acid

Four and one-half hours after the administration of 4.5 mg of retinoic acid to a rat with a cannulated bile duct, tissues were examined for free retinoic acid. Portions of fraction II from bile, liver, and small intestine were treated with diazomethane and analyzed by gas chromatography. The fraction eluted from gas chromatography with the retention time of all-*trans* methyl retinoate was collected, was dissolved in ethanol, and was examined spectrophotometrically. Radioactivity was measured in corresponding fractions and components.

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[5] R. D. Zachman, personal communication.

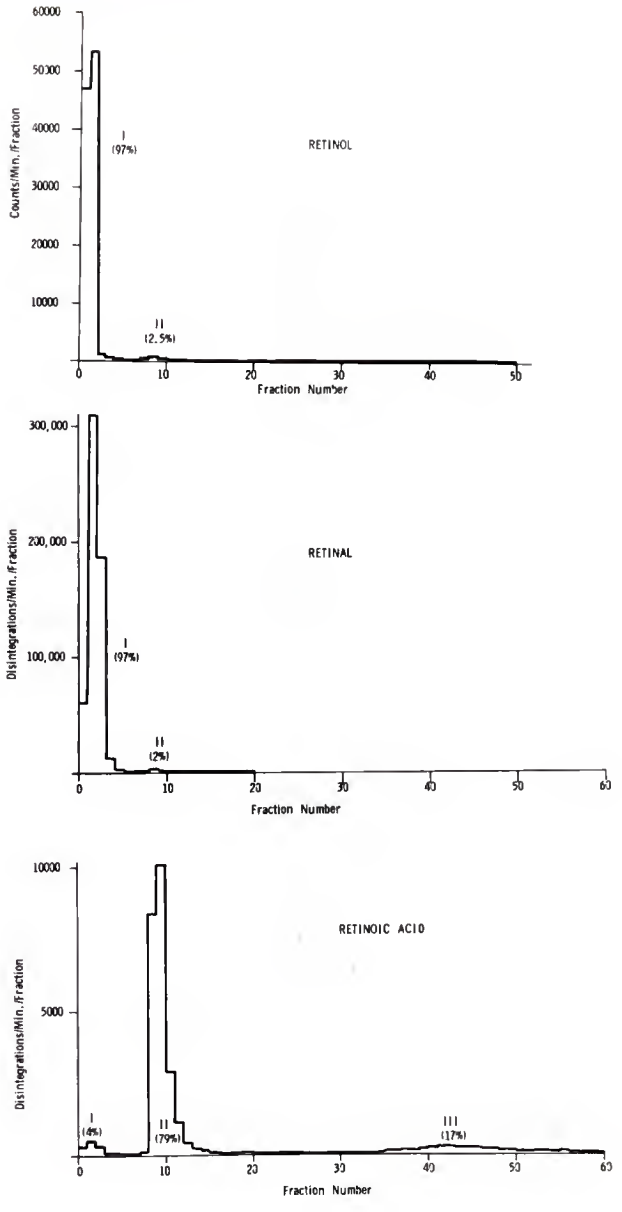

Fig. 13. Separation of a liver extract by ion exchange chromatography after administration of C^{14}-retinol, C^{14}-retinal, or C^{14}-retinoic acid. The liver extract, which was prepared as described in the text, was examined 24 hrs. after the administration of retinol, 6 hrs. after the administration of retinal and 4.5 hrs. after the administration of retinoic acid.

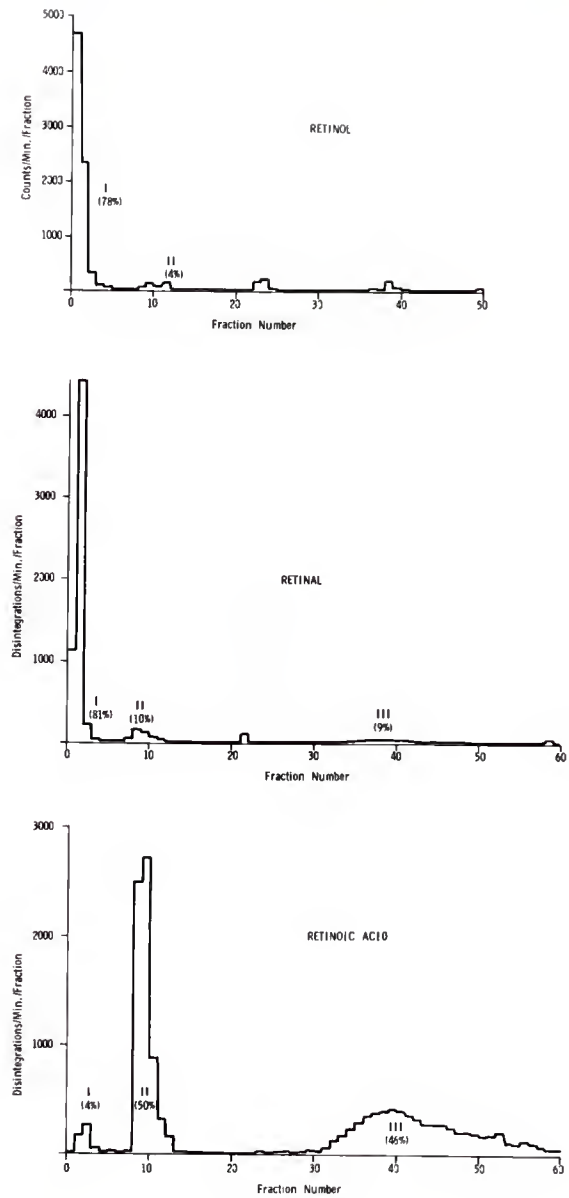

Fig. 14. Separation of an intestinal extract by ion exchange chromatography after administration of C^{14}-retinol, C^{14}-retinal, or C^{14}-retinoic acid. The intestinal extract, which was prepared as described in the text, was examined 24 hrs. after the administration of retinol, 6 hrs. after the administration of retinal, and 4.5 hrs. after the administration of retinoic acid.

The specific activity of retinoate found in fraction II was increased slightly by gas chromatographic purification to a value approximately equal that of the injected substrate (Table III). The absorption maximum of retinoic acid at 347 mμ was clearly evident in the ultraviolet spectra of fraction II of the small intestine and liver (Fig. 15), but was obscured in fraction II of bile by some material absorbing primarily at 400 mμ (Fig. 15). This material was also found in normal bile of untreated animals (Fig. 15).

Thus, free retinoic acid persisted in tissues of the rat for at least 4.5 hours after its intravenous injection. During this period the endogenous production of retinoic acid was small in comparison with the administered dose.

Characterization of Metabolites of Retinoate in Bile After the Administration of C^{14}-Retinoic Acid

Experimental procedure

Bile, which contained more radioactivity than any other source when investigated 6 hours after the administration of labeled retinoate, was carefully analyzed qualitatively and quantitatively by ion exchange, gas, and thin layer chromatography to ascertain the nature of all the radioactive metabolites of retinoic acid. The bile from six rats dosed with 2 to 3 mg each of labeled retinoic acid was pooled and examined according to the scheme shown in Fig. 16. The bile from six additional rats dosed similarly was also separated by ion exchange chromatography, and the resultant fraction III was further purified by silicic acid chromatography. This purified fraction III was treated with various reagents to determine the nature of its functional groups.

Table III

Specific activities of retinoic acid from various tissues 4.5 hrs. after the injection of 6,7-C^{14}-retinoic acid

Tissue	Radioactivity recovered in fraction II	Specific activity of fraction II from anion exchange chromatography	Specific activity of fraction II after gas chromatography
	% of initial dose	dpm/µg	dpm/µg
Original retinoate substrate	---	139	---
Liver	5.2	110	125
Small intestine	2.1	106	128
Bile	3.2	133	134

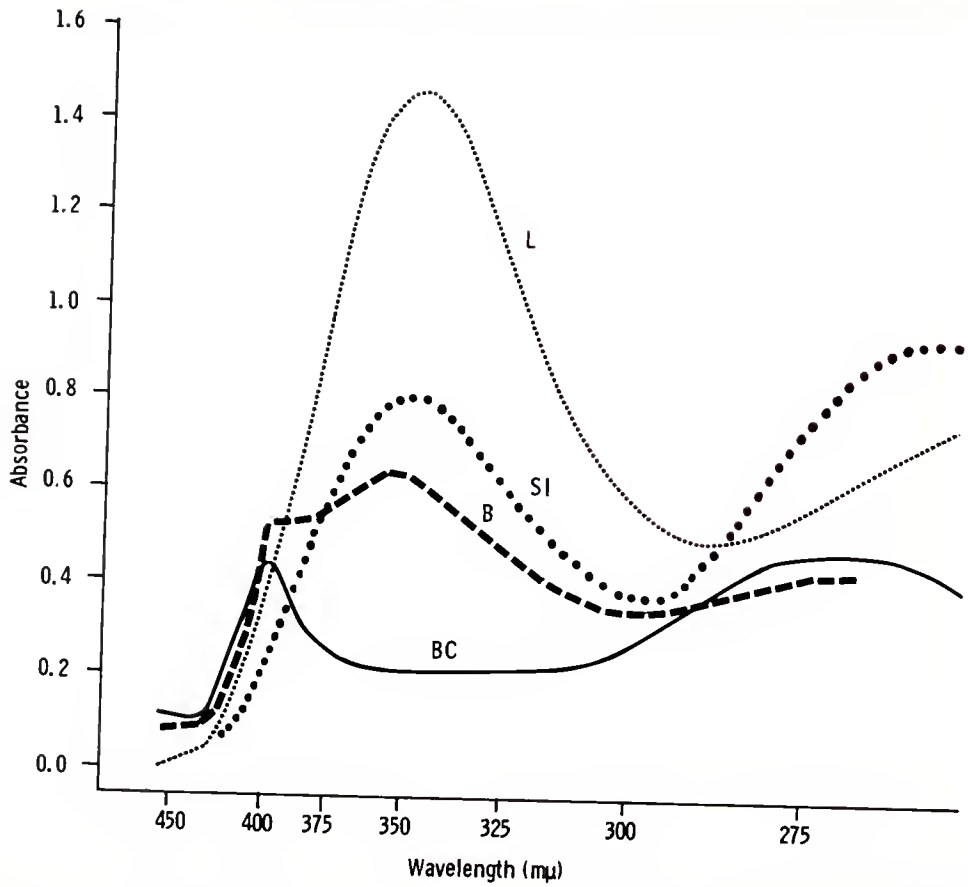

Fig. 15. Ultraviolet spectra of fraction II of bile, liver, and intestine 4.5 hrs. after the administration of retinoic acid.

B Bile

L Liver

SI Small intestine

BC Bile from a rat to which no retinoate was administered

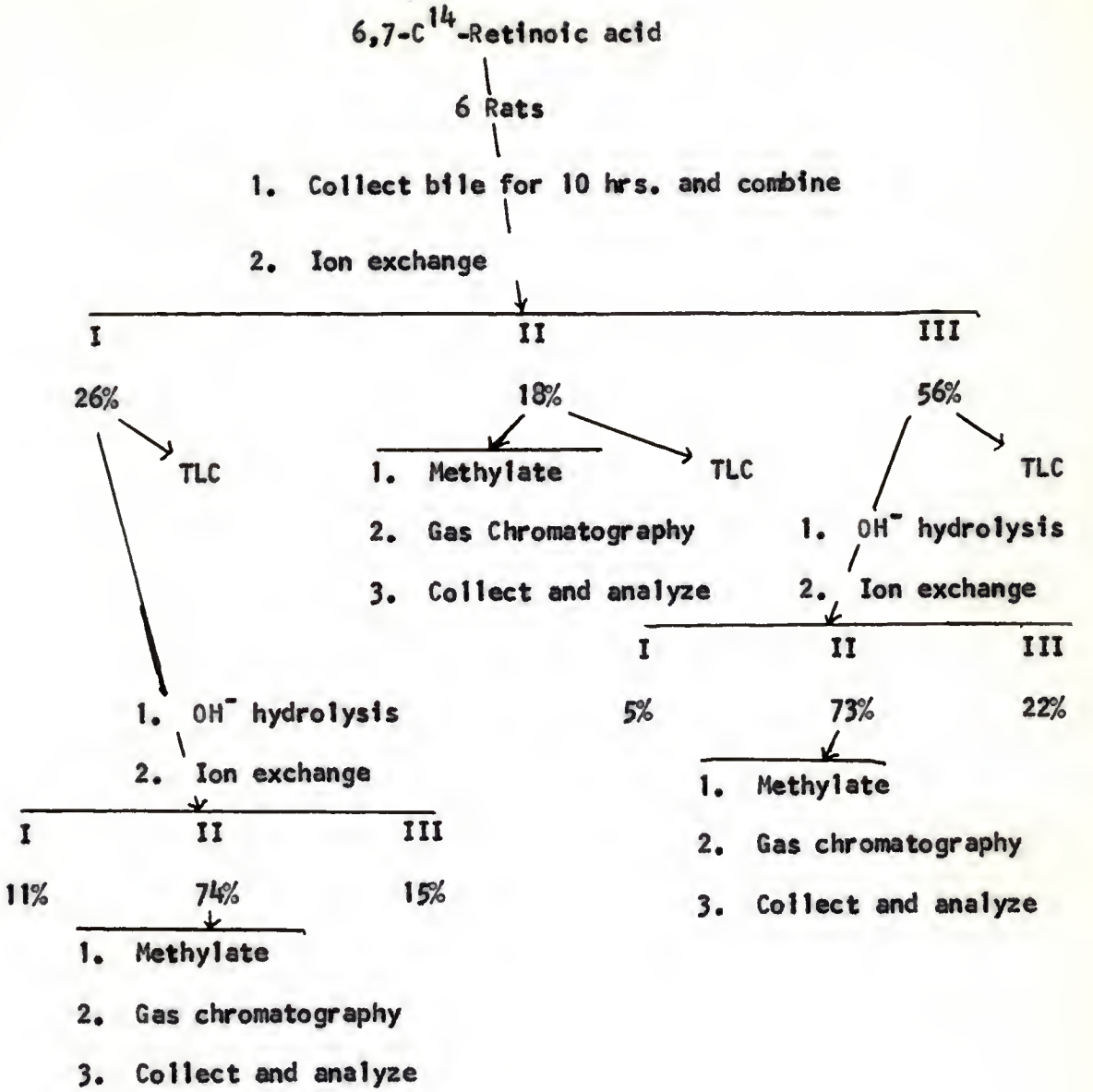

Fig. 16. Flow sheet for the treatment of bile after injection of 6,7-C^{14}-retinoic acid. Percentages refer to the relative amounts of radioactivity found in each fraction after ion exchange chromatography.

The bile, accumulated for up to 10 hours after the administration of radioactive retinoic acid to each of these twelve rats, was separated by ion exchange. Bile collected for longer periods of time was assayed for total radioactivity only, since it was relatively non-radioactive. Several organs from these twelve rats were also monitored for total radioactivity for period up to 96 hours after administration of retinoic acid.

The rate of disappearance of labeled retinoic acid from various tissues and its excretion in the bile

The total radioactivity found in the tissues of twelve bile duct cannulated rats after the administration of 2-3 mg of retinoic acid is shown as a function of time in Fig. 17. Radioactivity appeared rapidly in the liver, kidney, small intestine, and the bile. Thereafter, the radioactivity in the tissues decreased at similar rates. Within 96 hours virtually 100% of the injected dose of C^{14} was excreted in the bile. Apparently, the animal's entire store of injected retinoic acid is eventually transported to the liver and excreted in the bile, largely as a component of fraction III.

Fraction I of bile

Fraction I of bile, which contained the majority of the non-radioactive components of bile, was difficult to analyze. The characteristic absorption maximum of retinoic acid appeared in the ultraviolet spectrum of fraction I from treated animals but not in that from control animals (Fig. 18). Therefore fraction I of bile appeared to be an ester of retinoic acid. To test this hypothesis, fraction I was hydrolyzed with base, neutralized, and separated by ion exchange chromatography. Seventy-four percent of the recovered radio-activity was found in fraction II (Fig. 16). The ultraviolet absorption

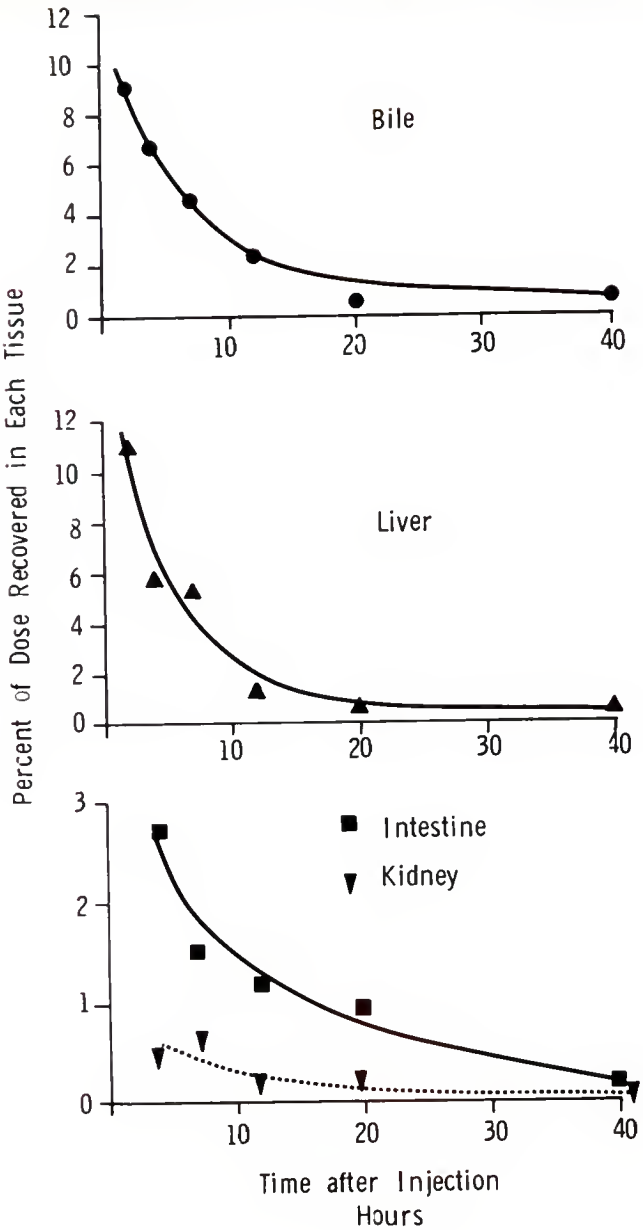

Fig. 17. Effect of time on the recovery of injected radioactive retinoic acid in various tissues. Two to three milligrams were administered to each of twelve rats and the cited tissues were analyzed for total radioactivity at the specified times.

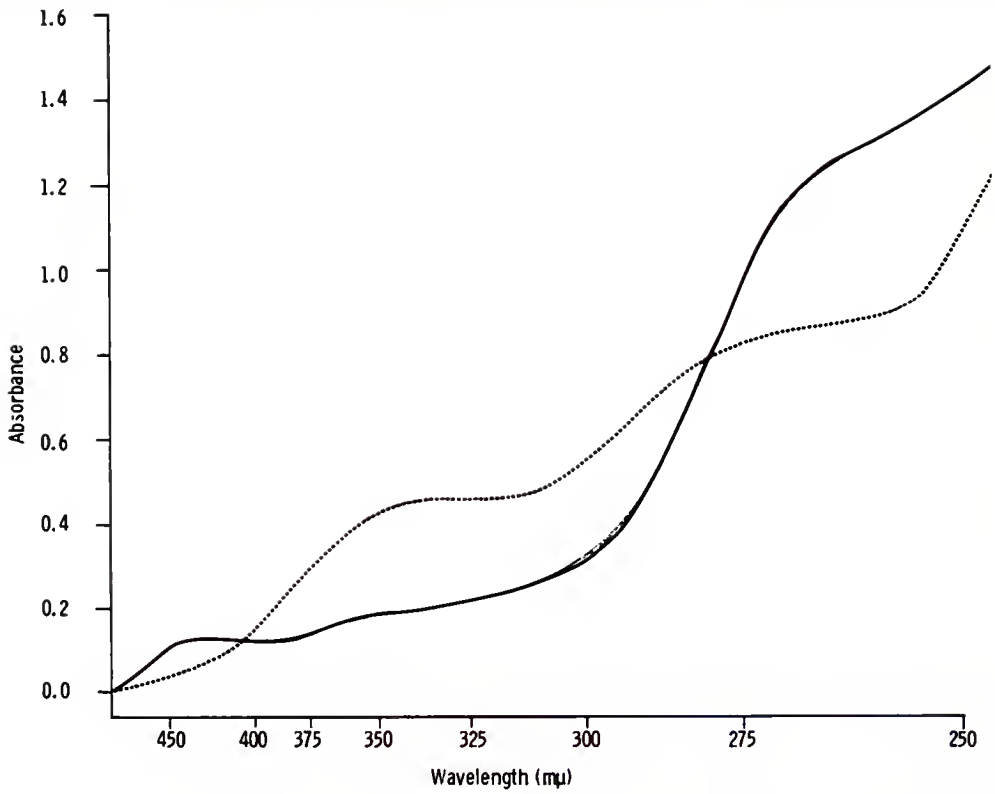

Fig. 18. Spectra of fraction I of bile.

..... Fraction I of bile collected for 10 hrs. after the administration of retinoic acid.

_____ Fraction I of bile obtained from a rat not injected with retinoic acid.

spectrum of this fraction was characteristic of retinoic acid, although it was partially obscured by material absorbing primarily at lower wavelengths.

Upon gas chromatography of the methylated basic hydrolysate of fraction I, several radioactive components appeared (Fig. 19). The retention times and absorption maxima of the two main radioactive peaks corresponded exactly to components B and C of standard methyl retinoate (Fig. 20). A third radioactive peak, apparently present in substrate retinoate (Fig. 9) but not in an isomerate of non-radioactive retinoic acid (Fig. 8), was situated between the last two retinoate isomers. This compound did not absorb appreciably at 350 mμ. All three components absorbed extensively at lower wavelengths, however, and were probably contaminated with components of fraction I which overloaded the column and slowly bled through it. Although a radioactive peak corresponding to component A appeared in the radiogram, so much other lipid material was eluted in the same fraction that spectral analysis was not worthwhile. By summation of the radioactivity in characteristic components of retinoate, retinoate isomers account for at least 25% of the C^{14} recovered upon gas chromatography of methylated fraction I (Table IV), confirming the hypothesis that fraction I contains an ester of retinoic acid.

The average specific activities of the retinoate in fraction I of bile before and after hydrolysis and of the retinoate isomers found after gas chromatographic analysis of fraction I hydrolysate were approximately four times higher than that of the substrate retinoic acid (Table IV). Since the specific activity of the cis isomers of the labeled substrate retinoic acid was much higher than that of the

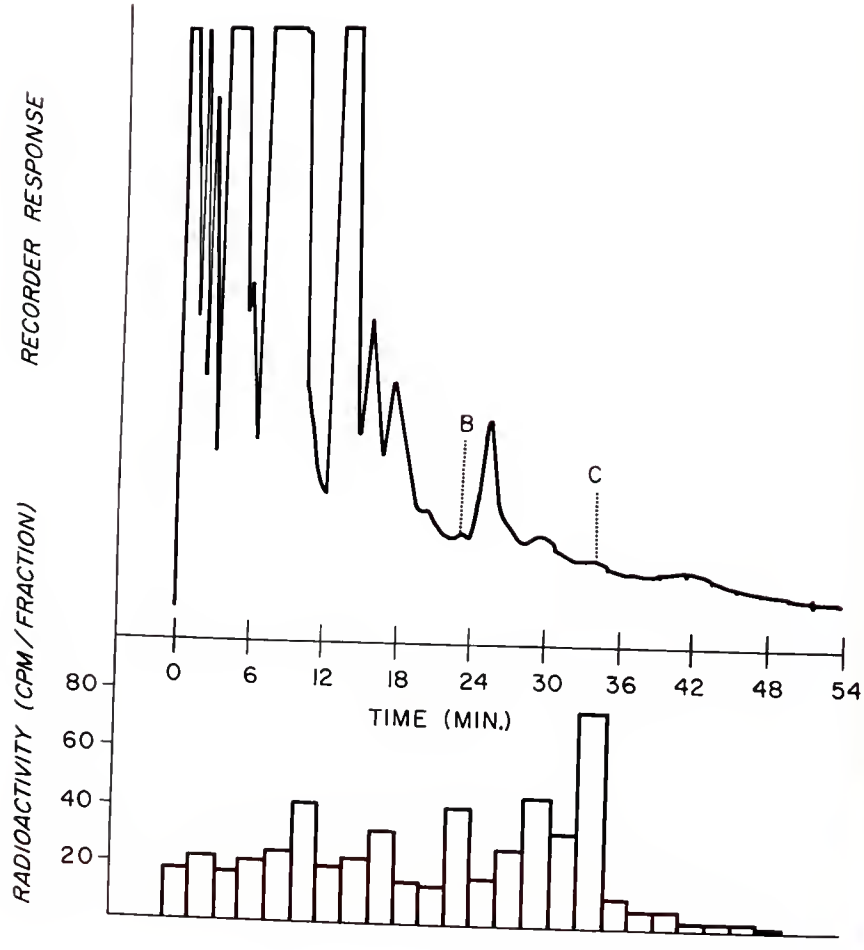

Fig. 19. Gas chromatography of fraction II obtained after the hydrolysis of fraction I of bile collected from rats injected with C^{14}-retinoic acid. Whole bile was chromatographed on an ion exchange column, the resultant fraction I was hydrolyzed with NaOH, neutralized, and rechromatographed on an ion exchange column. This final fraction II was methylated, and 10 µl were applied to gas chromatography. The upper trace shows the response of the argon detector, while the lower trace shows the corresponding radioactivity trapped on anthracene crystals.

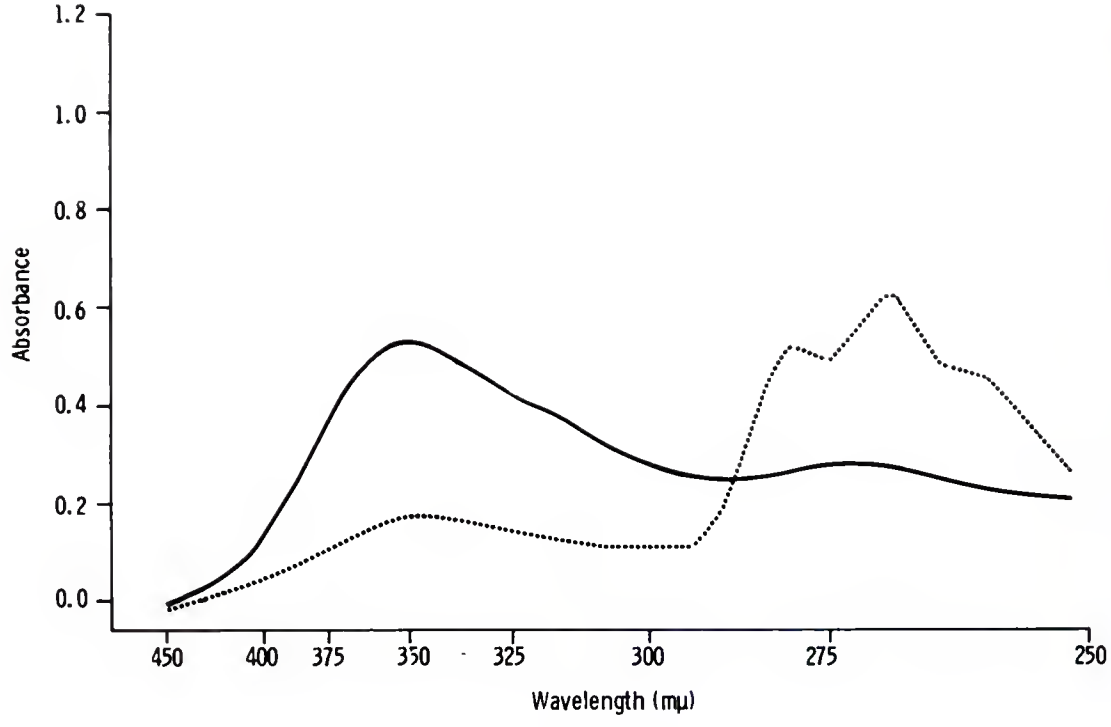

Fig. 20. Ultraviolet spectra of isomers of retinoate collected after gas chromatography of the methylated hydrolysate of fraction I.

..... Component B

_____ Component C

Table IV

Specific activities of 6,7-C^{14}-retinoic acid found in fractions of bile

After the administration of 6,7-C^{14}-retinoic acid, bile collected from six rats for 10 hrs. was pooled, chromatographed on an anion exchange column, hydrolyzed by base, methylated, and analyzed by gas chromatography. Since anion exchange fractions contained ultraviolet absorbing impurities, reported specific activites are minimum values. The percentage of the total recovered radioactivity which was found in each GLC fraction is given in parenthesis. Average deviations were calculated from duplicate assays.

Sample	Specific activities				
	Average initially	Average after basic hydrolysis	Methylated Components of GLC A	B	C
	dpm/μg				
Injected substrate	237±2	---	---	555 (15%)	190±17 (67%)
Fractions from anion exchange column					
I	>940	>970	-(3%)	2080±470 (6%)	1230±250 (17%)
II	>672	---	-(2%)	---(6%)	207±27 (21%)
III	>725	>762	---	861±1 (18%)	611±10 (26%)

all-*trans* isomer, the above results favor the direct biological esterification of *cis* isomers of retinoate without equilibration with the all-*trans* form.

Fraction II of bile

The characteristic absorption maximum of retinoic acid was virtually obscured in fraction II of this bile sample (Fig. 21), unlike the previously cited experiment (Fig. 15) in which the amount of injected substrate was greater. Upon gas chromatographic analysis, fraction II contained four radioactive peaks, the last three of which corresponded with peaks on the gas chromatogram (Fig. 22) and had the same relative retention times as components A, B, and C of methyl retinoate. Since the ultraviolet spectrum of collected componet C had the absorption maximum (352 mμ) of methyl retinoate (Fig. 21), the presence of retinoic acid in fraction II is confirmed.

Assuming that the last three radioactive peaks are isomers of retinoate, approximately 28% of the recovered radioactivity of fraction II consisted of retinoic acid (Table IV). Since fraction II was found to be grossly contaminated with fraction III in this case by TLC analysis, the reported content of retinoic acid is a minimal value. The specific activity of component C derived from fraction II of bile (207 $\frac{dpm}{\mu g}$) is approximately the same as that of the starting material (190 $\frac{dpm}{\mu g}$). Moreover, the relative distribution of retinoate isomers found in fraction II of bile is nearly the same as that present in the injected substrate. These findings suggest that retinoic acid was absorbed by the liver from the blood and directly excreted into the bile without appreciable isomerization or dilution by endogenous retinoic acid.

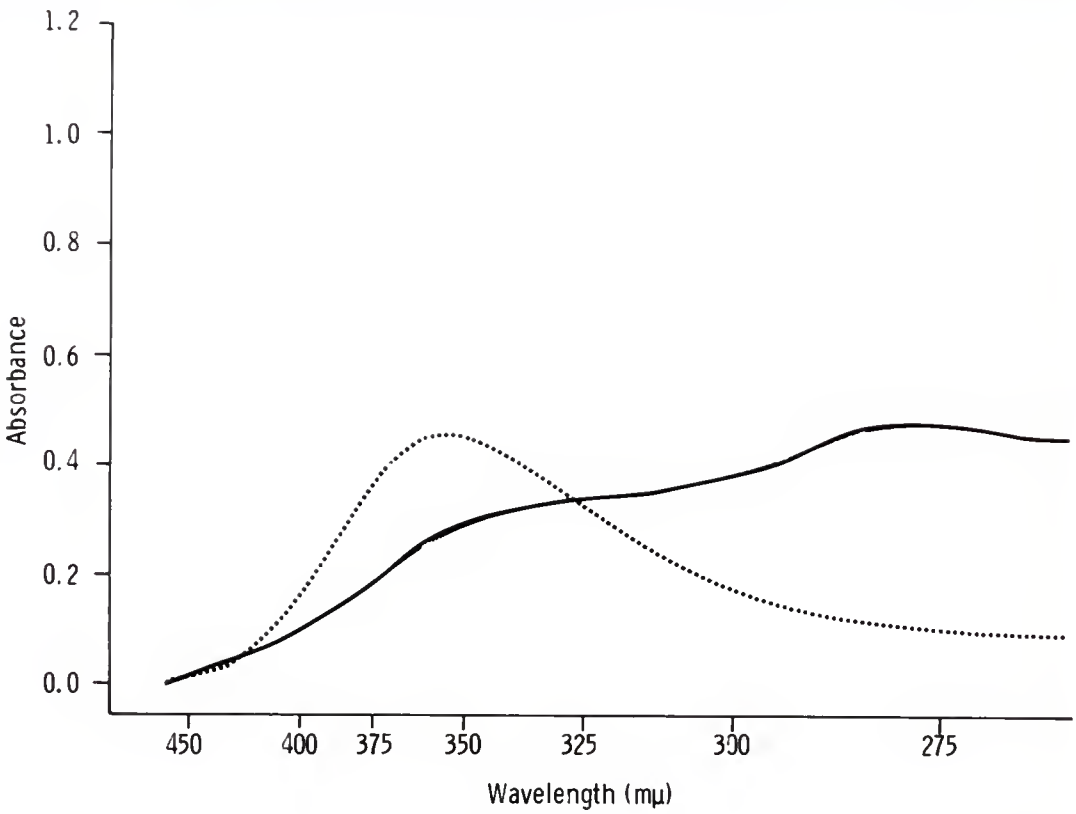

Fig. 21. The ultraviolet spectrum of fraction II of bile before and after gas chromatographic analysis.

_____ Spectrum of fraction II of bile

..... Spectrum of the major radioactive component (peak C) of methylated fraction II after gas chromatography

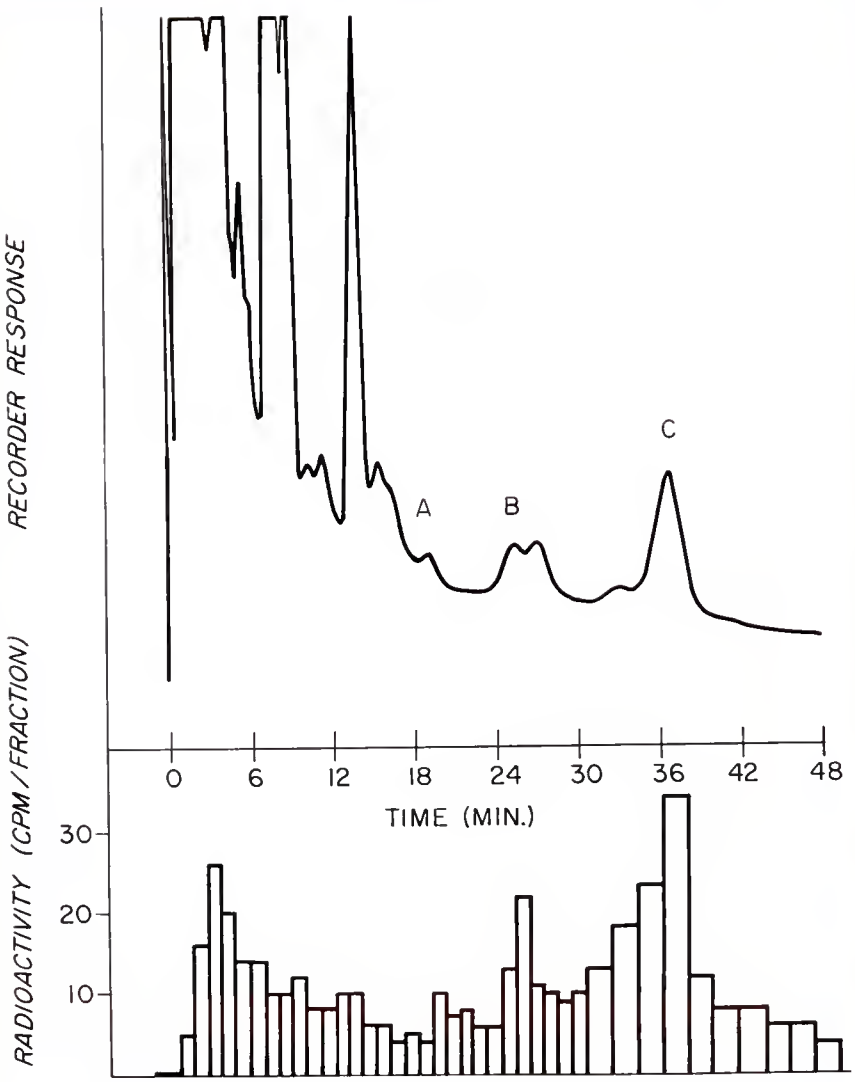

Fig. 22. Gas chromatography of fraction II from bile after the administration of C^{14}-retinoic acid. Whole bile collected from animals injected with C^{14}-retinoic acid was separated by ion exchange chromatography. The resultant fraction II was methylated and 10 µl were applied to the gas chromatograph. The top trace shows the response of the argon detector while the lower trace shows corresponding radioactivity collected on anthracene crystals.

Fraction III of bile

The ultraviolet spectrum of fraction III of bile clearly showed the absorption maximum (352 mμ) of retinoic acid (Fig. 23), indicating that fraction III must contain an acidic conjugate of retinoic acid. No radioactive components were detected by gas chromatography of fraction III, even after methylation and preparation of the silyl ether. Apparently, the volatility of the radioactive components in fraction III is low, perhaps due to their high molecular weight or polarity. After anion exchange chromatography of the basic hydrolysate of fraction III, however, 73% of the recovered radioactivity appeared in fraction II (Fig. 16). This fraction had the characteristic ultraviolet absorption maximum of retinoic acid.

Upon methylation and gas chromatography of this resultant fraction II, the two major peaks of mass and radioactivity which appeared (Fig. 24) corresponded exactly in retention times to components B and C of methyl retinoate. These two major components, which contained 18% and 26% of the recovered radioactivity, possessed the absorption maxima of retinoate isomers at 345 mμ and 353 mμ, respectively (Fig. 25).

A smaller radioactive component, which contained 13% of the recovered radioactivity, was also present in resultant fraction II. A similar component was also present in labeled substrate retinoic acid, but not in the retinoate isomerate (Fig. 7).

The calculated specific activity of retinoate in fraction III before hydrolysis, in fraction II after ion exchange chromatography of hydrolyzed fraction III, and in methyl retinoate after methylation and gas chromatography of the resultant fraction II was about four times

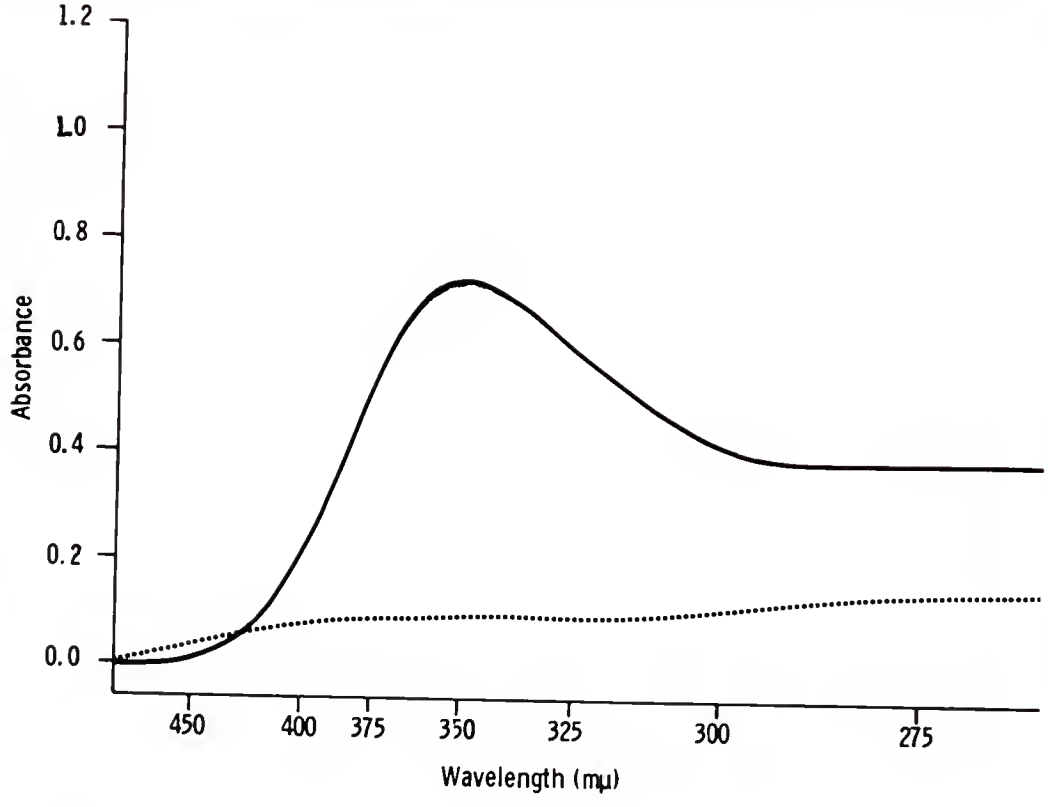

Fig. 23. Ultraviolet spectrum of fraction III of bile after administration of C^{14}-retinoic acid.

_____ Fraction III of bile after the administration of retinoic acid.

..... Fraction III of bile from a rat not injected with retinoic acid.

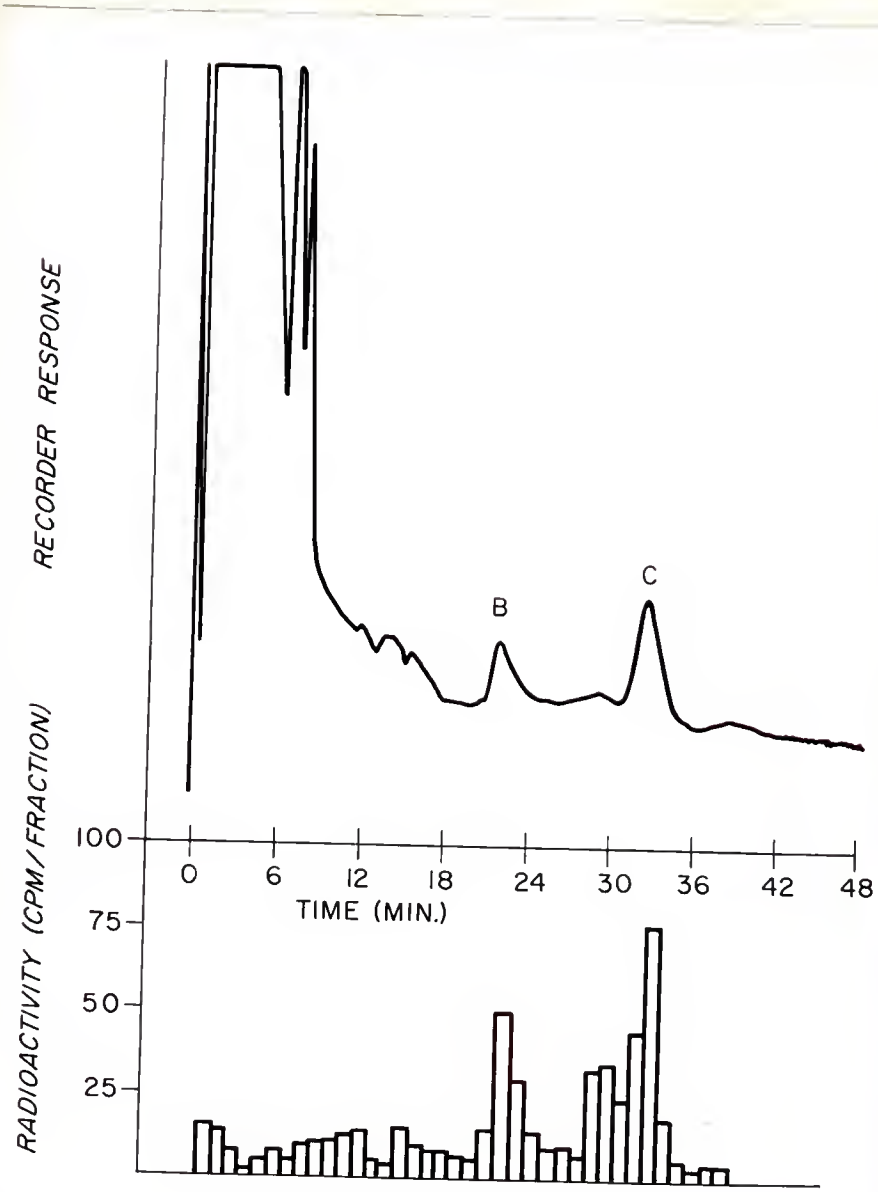

Fig. 24. Gas chromatography of methylated fraction II obtained by ion exchange chromatography of a basic hydrolysate of fraction III of bile from a rat treated with C^{14}-retinoic acid. Whole bile collected from animals injected with C^{14}-retinoic acid was separated by ion exchange chromatography. The resultant fraction III was hydrolyzed with NaOH and separated on an ion exchange column. The resultant fraction II was methylated, and 10 µl were applied to the gas chromatograph. The upper trace shows the response of the argon detector while the lower trace shows the corresponding radioactivity collected on anthracene crystals.

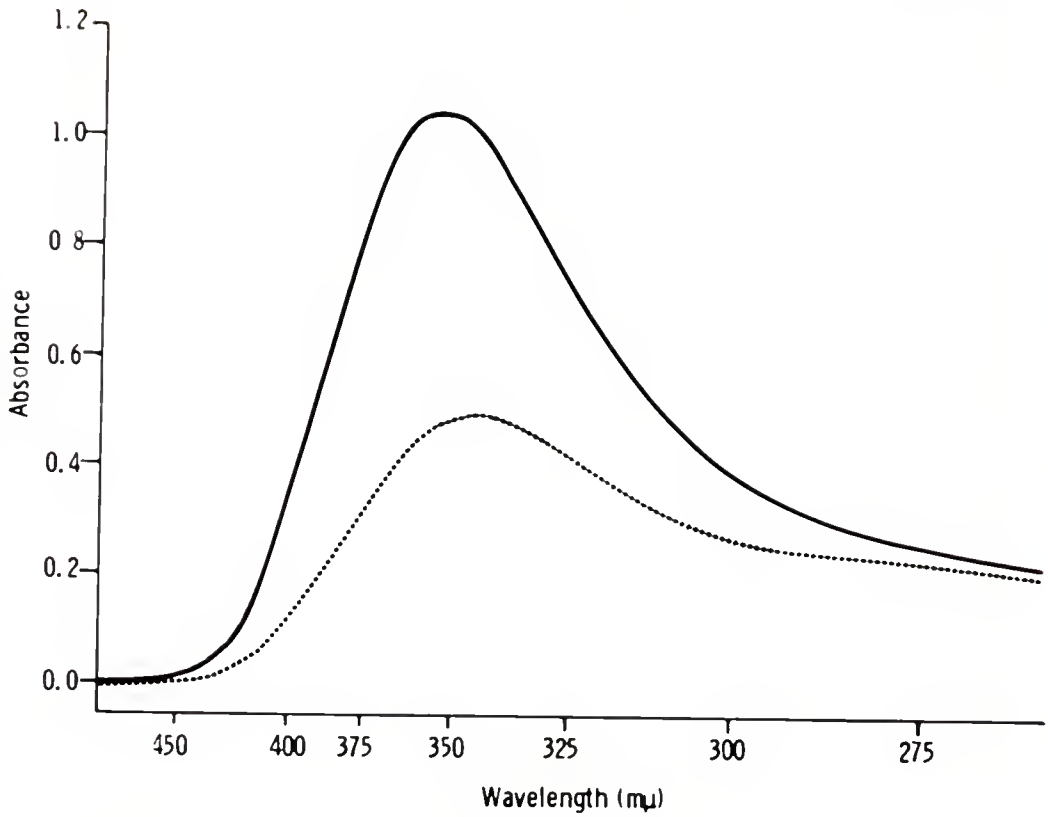

Fig. 25. Ultraviolet spectrum of retinoate isomers collected from gas chromatography of methylated fraction III hydrolysate.

..... Component B

_____ Component C

that of the injected labeled substrate retinoic acid (Table IV). As in the case of fraction I, isomers of substrate retinoate are apparently incorporated into the metabolites of fraction III without prior equilibration with the all-trans isomer.

A minimum of 57% of the recovered radioactivity from fraction III consisted of retinoic acid. Since no other obvious radioactive peaks appeared upon gas chromatography of the methylated hydrolysate of fraction III, the radioactivity found in fraction III of bile after the administration of 2-3 mg of C^{14}-retinoic acid seemingly resides almost exclusively in an acidic conjugate, or conjugates, of retinoic acid.

Investigation of bile by thin layer chromatography (TLC)

Bile from six rats dosed with 2-3 mg of C^{14}-retinoic acid was separated by ion exchange chromatography. The resulting fractions were further separated by three different TLC systems. The distribution of radioactivity in each sample was obtained by counting sequential samples of silica gel scraped in 0.5 cm increments along the migration path. The R_f values of the major radioactive maximum of the components on the thin layer chromatograms are listed in Table V.

When fractions III and whole bile were developed with water, the predominant radioactive maxima were associated with fluorescent spots with an R_f of 0.75. In the same system, the R_f of radioactive maxima from fractions I and II and of retinoic acid was zero.

When developed with benzene:chloroform:methanol (4:1:1) (Fig. 26), a relatively non-polar solvent system, the R_f of the major radioactive maximum of fraction I was about 0.75, that of fraction II was 0.20, and that of whole bile and fraction III was only 0.03. The radioactive maximum of fraction III, which contains relatively few components, also

Table V

Thin layer chromatography of rat bile 10 hrs. after administration of C^{14}-retinoic acid

After separation of various bile fractions by TLC, uniform increments of the adsorbant were scraped into counting vials and assayed for radioactivity by liquid scintillation spectrometry. The relative amounts of radioactivity in the radioactive maxima of each sample are listed with the corresponding R_f's.

Sample	Developing system					
	H_2O		Benzene:chloroform: methanol (4:1:1)		Benzene:chloroform methanol:acetic acid (5:5:5:1)	
	R_f	%	R_f	%	R_f	%
Whole bile	0.00	10	0.02-0.15	60	0.44	60
	0.55-0.75	60	0.84-0.92	10	0.87	10
					0.97	10
Fraction I	0.00	60	0.08	10	0.25	35
			0.72	40	0.93	50
			0.84	20		
Fraction II	0.02	20	0.20	70	0.05	15
					0.80	70
Fraction III	0.15-0.40	20	0.02-0.10	80	0.46	60
	0.70-0.85	35				
Retinoic acid	0.00		0.34		0.91	

Fig. 26. Thin layer chromatographic comparison of whole bile and fractions of bile obtained by ion exchange chromatography. Samples were developed in benzene:chloroform:methanol (4:1:1), detected by I_2 vapors, and photographed through a blue filter. Samples were:

1 and 6	5 µg of retinoic acid
2	Fraction I
3	Fraction II
4	Fraction III
5	Whole bile

fluoresced under ultraviolet light like retinoic acid and stained with iodine vapor. The R_f of the radioactivity in fraction II was invariably lower (0.20) than that of retinoic acid (0.34), but corresponded to an artifact of all-trans retinoic acid which usually occurred after exposure of the plate during application of sample.

Modification of the previous developing system by adding acetic acid and reducing the amount of benzene increased the migration rate of all components (Table V). The major radioactive components of fractions I and II migrate near the solvent front, whereas the R_f of radioactive maxima in fraction III and whole bile is about 0.5. The radioactivity of fraction III was associated with a fluorescent area (R_f 0.44) which apparently consisted of two components.

The following information was obtained about the composition of fractions I, II, and III as a result of thin layer chromatographic analysis. (a) Adequate separation of whole bile and fractions I and II is difficult due to the presence of a large amount of non-radioactive material. On the other hand, fraction III, which has fewer components, was separated well on TLC. (b) Fraction I is composed of at least two radioactive compounds, a water insoluble, non-polar component, and a somewhat more polar component. (c) The radioactive maximum of fraction II always corresponded to an artifact in standard all-trans retinoic acid when analyzed by the benzene:chloroform:methanol (4:1:1) system. Apparently the small amount of retinoic acid on the plate is oxidized during exposure to air and light and thus migrated differently from the standard retinoate. (d) The major radioactive component of fraction III was water soluble, was very polar, and fluoresced like retinoic acid when irradiated by ultraviolet light. (e) The radioactivity of whole

bile largely consisted of fraction III, which is in agreement with the results of ion exchange chromatography.

Purification and characterization of fraction III of bile by chromatography on silicic acid

When fraction III, obtained by ion exchange chromatography of pooled bile, was chromatographed on a column of silicic acid, three major radioactive components were found (Fig. 27). The first component absorbed ultraviolet light non-specifically, but when analyzed by TLC (Fig. 28), contained a radioactive substance with the R_f of free retinoic acid as well as several other substances. The second and third radioactive components from the silicic acid column possessed the absorption spectrum of retinoic acid with maxima at 345 and 355 mμ, respectively, and contained the major portion of the radioactivity in fraction III. Upon thin layer chromatographic separation and iodine staining, no major impurities were evident (Fig. 28). Thus separation of fraction III by silicic acid chromatography removed much of the impurities including some free retinoic acid.

In an attempt to determine the nature of the functional groups of the retinoic acid conjugate of fraction III, several derivatives of purified fraction III were analyzed by thin layer chromatography. Fraction III, purified on a silicic acid column, was first treated with diazomethane, which would make methyl esters of any acidic groups, and then with hexamethyldisilazane in pyridine, which would make ethers of any free hydroxyl groups. Upon comparison of these derivatives with untreated fraction III by thin layer chromatography (Fig. 29), the R_f values and relative rates of migration were ester-ether (0.78)>ester (0.29)>untreated fraction III (0.03). Retinoic acid in the conjugate was unaffected by this treatment, since the radioactivity maximum and

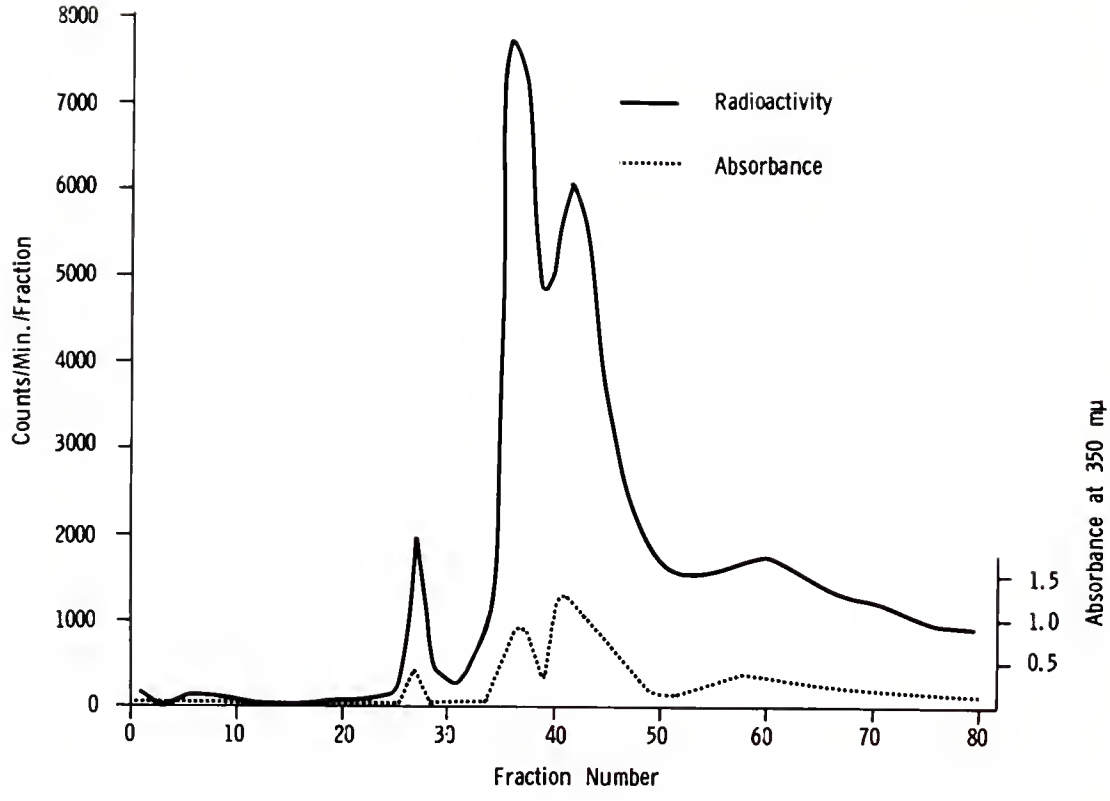

Fig. 27. Silicic acid chromatography of fraction III of bile after the administration of C^{14}-retinoic acid. The gradient elution solvent system is described in the methods section.

_____ Radioactivity

..... Optical density at 350 mμ

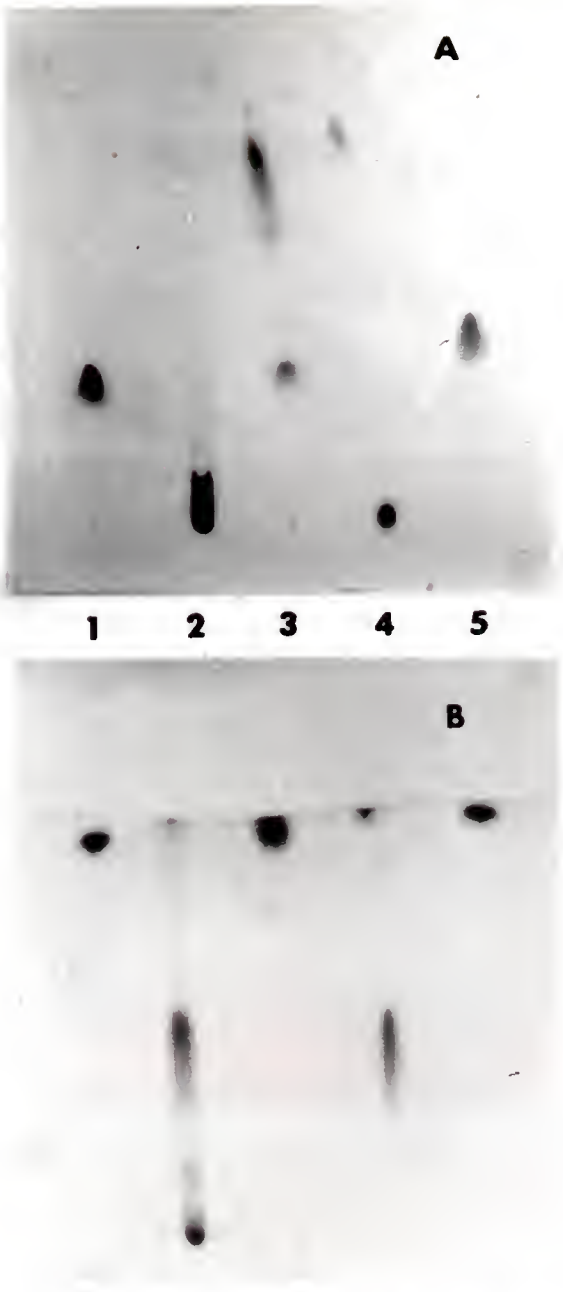

Fig. 28. Thin layer chromatography of components isolated by silicic acid chromatography of fraction III from bile. The developing system in A was benzene:chloroform:methanol (4:1:1), and in B was benzene:chloroform:methanol:acetic acid (5:5:5:1). Samples were:

1, 5 Retinoic acid

2 Fraction III unpurified

3 Eluate from fraction 27

4 Eluate from fractions 35-50

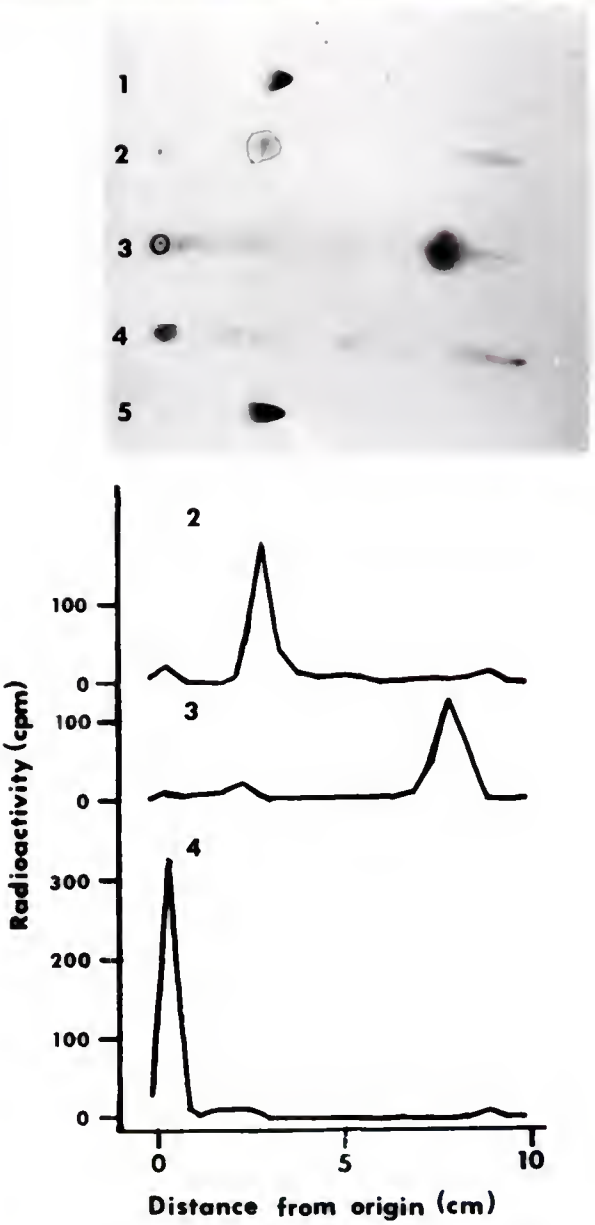

Fig. 29. Behavior of derivatives of purified radioactive fraction III which were separated on thin layer plates of Silica Gel G by development with benzene:chloroform:methanol (4:1:1). A photograph of the iodinated plate is given in the upper figure, and the distribution of radioactivity in various compounds is given below. Samples were:

1,5 Non-radioactive retinoic acid

2 Fraction III, methyl ester

3 Fraction III, silyl ether of the methyl ester

4 Fraction III, untreated

fluorescence exactly coincided in each of the three spots. Evidently the non-radioactive moiety of the fraction III conjugate possesses a carboxyl group or other acidic function and one or more hydroxyl groups.

Since glucuronides of several lipid alcohols and acids are formed in biological systems, the corresponding derivatives of glucuronic acid were analyzed in the identical manner. Glucuronic acid, its methyl ester, and the ether of its methyl ester migrated on thin layer plates with R_f values of 0.00, 0.07, and 0.80, respectively. Whereas the methyl glucuronate tetrasilyl ether and the methyl ester-ether of fraction III migrated similarly, methylated fraction III was less polar than methyl glucuronate, and the untreated fraction III was slightly less polar than free glucuronic acid. On structural grounds a conjugate of retinoic acid and glucuronic acid would behave similarly to glucuronic acid; therefore the behavior of fraction III is consistant with a hypothetical conjugate of retinoic acid and glucuronic acid.

Characterization of Retinal Metabolites

Fraction I of bile

Fraction I obtained from ion exchange chromatography of rat bile after administration of 3 mg of C^{14}-retinal failed to show the spectrum of retinal or retinol ester. Apparently neither the substrate nor the storage formed of vitamin A is excreted in the bile. Further studies were not conducted because of the small amount of radioactivity found in this fraction.

Fraction II of bile

Fraction II did not possess the characteristic absorption spectrum of retinoic acid. After the addition of 5 mg of non-radioactive 95% all-trans retinoic acid to fraction II, retinoic acid was crystallized four times to a constant specific activity. The fourth crystals had a specific activity 8.5% that of the original material (Table VI). A non-radioactive impurity, which co-crystallized with retinoic acid, absorbed slightly at 345 mμ and thereby lowered somewhat the specific activity of the final crystals. The specific activity of the supernatant solution from the fourth crystallization, which was free from this impurity, was 11.5% that of the original material (Table VI). Upon gas chromatographic analysis of the final crystals, 15% of the recovered radioactivity appeared in component B and 85% in C. The specific activity of the collected methyl ester of component C agreed with that of the final supernatant solution (Table VI).

Since the crystals failed to account for most of the radioactivity in fraction II, supernatant solutions from the first three crystallizations were combined, methylated, and analyzed by gas chromatography. Qualitatively the same peaks occurred in the chromatogram of the combined supernatant solutions as in those of the final crystals (Fig. 30). Of the recovered radioactivity, 13% appeared in retinoate component B and 23% in component C. The average weighted specific activity of collected methyl retinoate isomers was 17.5 $\frac{dpm}{\mu g}$, or 53% of the specific activity of the original solution (Table VI).

In summary 53% of the radioactivity in fraction II of bile after C^{14}-retinal administration apparently consisted of retinoic acid, of which 8.5-11.5% was the all-trans isomer and 35-40% were other isomers.

Table VI

Specific activity of retinoic acid found in fraction II of bile 8 hrs. after injection of 6,7-C^{14}-retinal

Material analyzed	Specific activity	Total retinoic acid present
	dpm/µg	mg
Original fraction II diluted with non-radioactive retinoic acid	33.0	5.15
1st Crystallization	4.4	2.98
2nd Crystallization	3.1	2.18
3rd Crystallization	3.2	1.74
4th Crystallization	2.8	1.06
4th Crystals		
Component C by GLC	3.8	
4th Supernatant solution	3.8	0.42
Combined supernatant solutions 1-3		
Component B by GLC	36.4	
Component C by GLC	12.9	

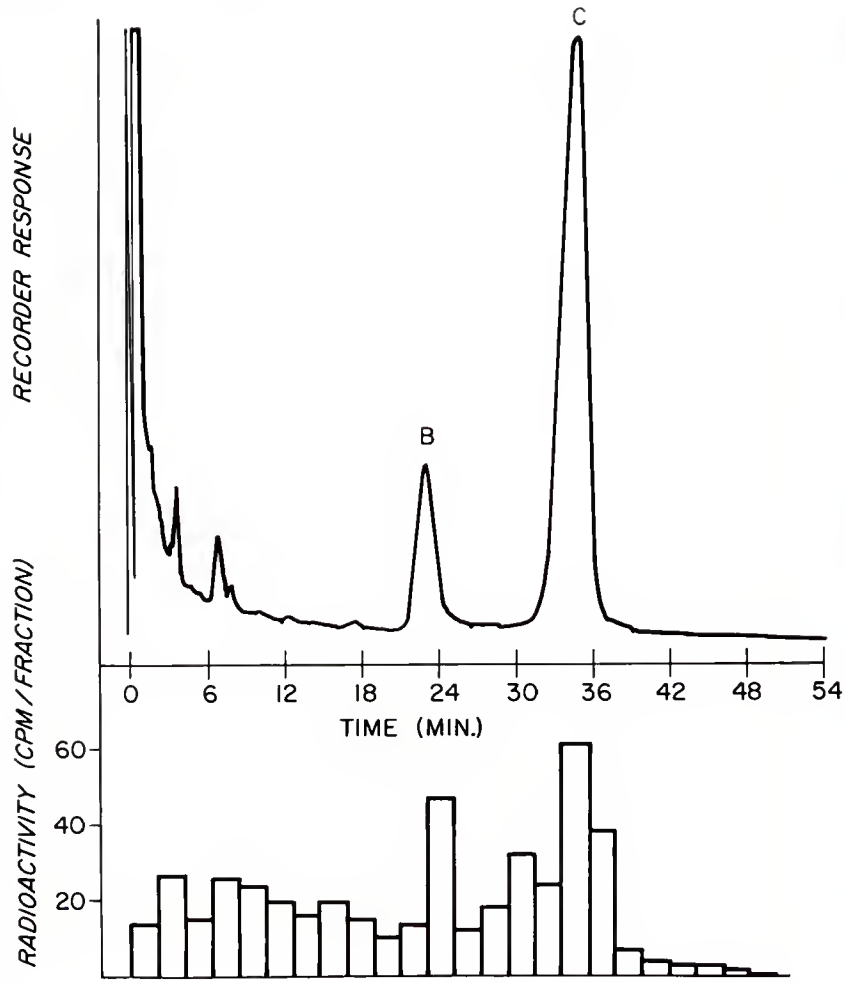

Fig. 30. Gas chromatography of fraction II obtained by ion exchange chromatography of bile after the administration of C^{14}-retinal. Whole bile collected from animals injected with C^{14}-retinal was separated by ion exchange chromatography. Fraction II so obtained was diluted with non-radioactive retinoic acid, was methylated, and 10 µl were applied to the gas chromatograph. The top trace shows the response of the argon detector, while the lower trace shows corresponding radioactivity collected on anthracene crystals.

Whether this distribution in the isomers has biological significance or only reflects isomerization during analysis has not been ascertained.

Fraction III of bile

To ascertain whether fraction III of bile contained conjugated retinoic acid after administration of C^{14}-retinal, fraction III was hydrolyzed with base followed by ion exchange chromatography. Seventy percent of the radioactivity in the hydrolysate was extracted with methanol and of this methanolic extract, 53% of the radioactivity recovered from ion exchange chromatography was eluted in fraction II. The retinoate spectrum, although obscured by ultraviolet absorbing compounds in unhydrolyzed fraction III, was clearly evident in this resultant fraction II. After crystallization of this resultant fraction II with 5 mg of non-radioactive 95% all-trans retinoic acid, the specific activity of the crystals after crystallization was reduced to less than 5% of that found in the original diluted fraction II (Table VII). Essentially the same low specific activity was found in the methylated crystals purified by GLC.

Upon gas chromatography of the combined supernatant solutions from the first four crystallizations, the two major radioactive peaks, which contained 10% and 13%, respectively, of the recovered radioactivity, corresponded exactly to components B and C of added non-radioactive retinoic acid (Fig. 31). On the basis of data given in Table VI, the average specific activity of these two peaks was calculated to be 2.2 $\frac{dpm}{\mu g}$. Since the specific activity of the original solution was 10 $\frac{dpm}{\mu g}$, 22% of the radioactivity of fraction III was retinoic acid, in close agreement with the recovery data which indicated that 23% of fraction III was retinoic acid. Apparently fraction III as well as fraction II

Table VII

Specific activity of retinoic acid found in fraction III of bile 8 hrs. after injection of 6,7-C^{14}-retinal

Material analyzed	Specific activity	Total retinoic acid present
	dpm/μg	mg
Fraction III after hydrolysis, rechromatography on ion exchange, and dilution of the resultant fraction II with non-radioactive retinoic acid	10.1	5.02
1st Crystallization	4.24	2.84
2nd Crystallization	1.69	2.25
3rd Crystallization	0.78	1.59
4th Crystallization	0.60	1.00
5th Crystallization	0.40	0.67
5th Crystals		
Component C by GLC	0.34	
5th Supernatant solution	0.87	0.17
Combined supernatant solutions 1-4		
Component B by GLC	11.5	
Component C by GLC	1.4	

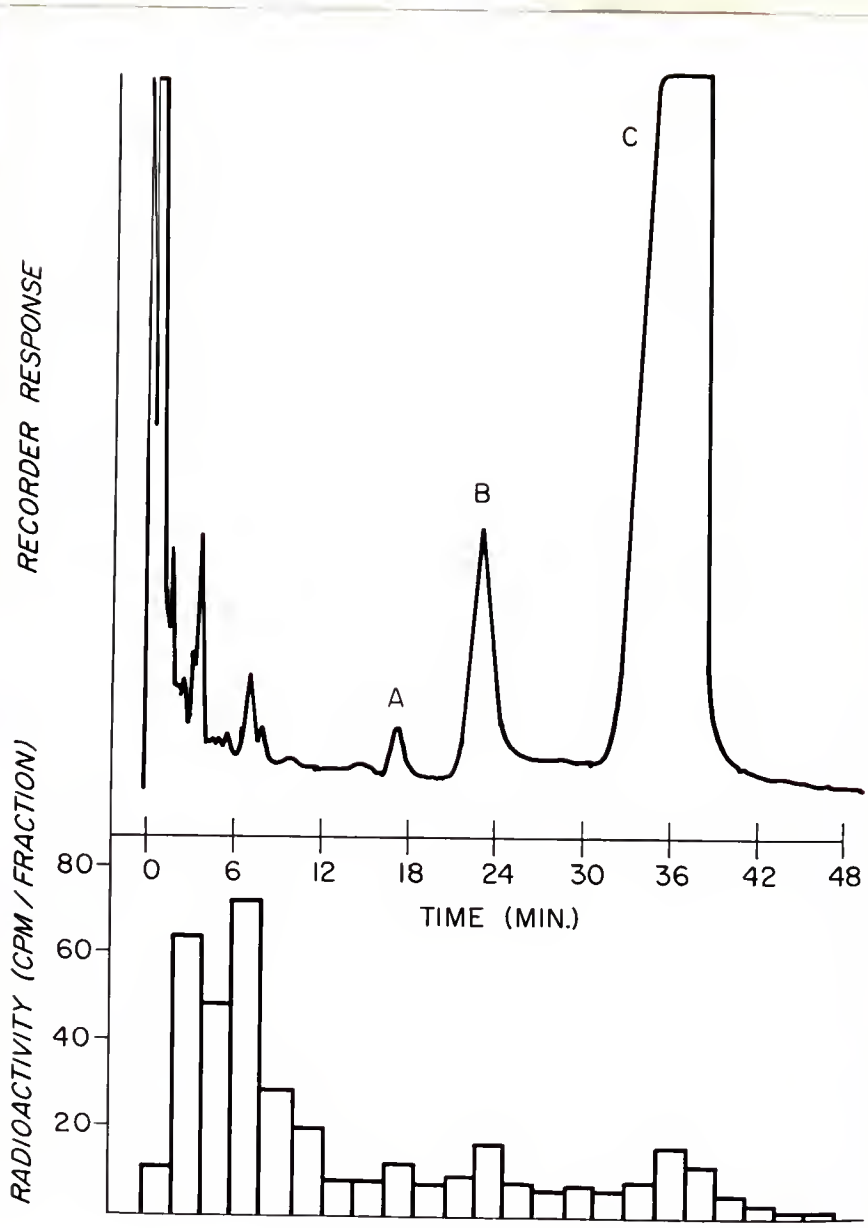

Fig. 31. Gas chromatography of fraction II obtained by ion exchange chromatography of the basic hydrolysate of fraction III after administration of C^{14}-retinal. Whole bile collected from animals injected with C^{14}-retinal was separated by ion exchange chromatography. Fraction III so obtained was hydrolyzed with NaOH, neutralized, and rechromatographed on an ion exchange column. The resultant fraction II was diluted with non-radioactive retinoic acid, methylated, and 10 µl were applied to the gas chromatograph. The upper trace shows the response of the argon detector, while the lower trace shows the corresponding radioactivity collected on anthracene crystals.

contain isomers of retinoic acid in addition to the all-*trans* form. In fraction III the radioactivity consisted of about 4-5% of all-*trans* retinoate and about 18% of other isomers.

DISCUSSION

Methods for detecting small amounts of retinol derivatives in biological tissues generally depend on the unique spectral properties of the conjugated alkene linkage. The absorption maximum is read either directly or after reaction with antimony trichloride or similar reagents (70). Because these assays are relatively non-specific and are affected by interfering chromophores (71), retinol derivatives are often purified before analysis. Several procedures have been employed: (a) differential solubility between various organic solvents, (b) adsorption chromatography on columns (72) or thin layer plates (51) of alumina or silicic acid, and (c) partition on liquid-liquid partition columns (73).

Since these methods separate compounds with relatively poor resolution on the basis of their polarity, solubility, and adsorption tenacity, retinol fractions are invariably contaminated with large quantities of other lipids. Thin layer chromatography has the additional disadvantage of exposing retinol derivatives to air and light during application of the sample and development of the plate (51).

Gas liquid chromatography, although of great utility in analyzing many lipids, has not previously been applied successfully to vitamin A. Ninomiya, in the only published report on the use of this method, noted extensive dehydration of retinol and its esters to anhydro retinol upon analysis by gas liquid chromatography (53). Because of the high sensitivity and resolution of this technique, however, further

study of its possible applicability to retinol analysis was deemed worthwhile. In the present investigation, factors which influence the behavior of retinol derivatives on GLC columns were carefully examined, and suitable procedures were developed for the isolation of several retinol derivatives with little destruction.

Many problems were posed in applying this technique to vitamin A. Although retinol derivatives can be volatilized, their instability imposes narrow limitations on the conditions which can be used. The conjugated alkene linkage, characteristic of retinol derivatives, is susceptible to free radical or acid catalyzed polymerization, isomerization, oxidation, and addition reactions (74). Moreover, compounds having a hydroxyl group alpha to an unsaturated bond easily undergo acid catalyzed dehydration (59, 75). High temperatures accelerate the rate of these reactions. Hence, the extensive formation of anhydro retinol observed by Ninomiya (53) is expected from a consideration of the chemical properties of this class of substances.

In order to minimize the destruction of retinol derivatives during gas chromatographic analysis, rapid elution of compounds at low temperatures was necessary. By employing low levels of liquid loading (1% SE-30), high flow rates (150 ml/minute), and short columns (2 ft.), short retention times for retinol derivatives were obtained. Destruction of retinol derivatives was further reduced by use of a solid support (Gas Chrom P) which was base washed to remove acidic sites and silanized to decrease the number of adsorptive sites. Under properly selected conditions anhydro retinol, methyl retinyl ether, retinal, and methyl retinoate were successfully separated by gas chromatography. In this system, however, retinyl acetate and retinol

were largely dehydrated. By pretreating the column with an antioxidant such as β-carotene or hydroquinone and by increasing the flow rate six fold, these compounds were also separated with little destruction. β-Carotene proved to be the most useful antioxidant since it remained on the column for several days, whereas hydroquinone exerted its protective effect for only an hour or two. Thus by eliminating conditions which promoted acid and free radical catalyzed reactions, even the more unstable retinol derivatives were separated by gas chromatography.

Unfortunately the efficiency of these columns was low. By increasing the concentration of the liquid phase, SE-30, to 3%, destructive, adsorptive sites on the column were further reduced. This column could be lengthened to 4.5 ft. and operated at a temperature of 180° and at a flow rate of 50 ml/minute without destruction of methyl retinoate, the most stable of the retinol derivatives tested. The column efficiency was therby increased from 250 to 1000 theoretical plates, sufficient for the separation of methyl retinoate from most other components, if not all, in tissue extracts.

Although the acidic character of some metabolites of retinoic acid has been recognized (41), anion exchange resins have not previously been employed for their purification. The preponderance of tissue lipids are non-ionic, however, and this technique allows the separation of small amounts of anionic compounds from the great mass of tissue lipids. Although acetic acid used in the eluant tended to catalyze the isomerization of retinoic acid, this effect was minimized by evaporating the acidic solvent in the dark at a low temperature <u>in vacuo</u>.

Thus, the procedure of chromatographing tissue extracts on anion exchange columns followed by chemical treatment of individual fractions and ultimately by gas chromatography of methylated derivatives was of critical importance in tracing the metabolism and fate of vitamin A derivatives in the rat.

Like retinol, retinoic acid supports growth (14-16), although it cannot be reduced to retinol in vivo. Presumably retinol is oxidized through retinal to retinoic acid, which then exerts a biological effect. Surprisingly, free retinoic acid has not been detected in tissues in vivo as a product of administered radioactive retinol, even when non-radioactive retinoic acid was also given as a trapping agent (22, 41). In those studies the lipid extracts were analyzed by thin layer chromatography. In view of our results, this negative finding is probably due to the use of a method which caused destruction of retinoic acid, and in addition was not adequately sensitive to detect the small amount of retinoic acid present in the liver and small intestine. In the present study, for example, the spectrum of retinoate was even obscured in crude fraction II after separation of the great mass of lipids from the acidic fraction of bile by ion exchange chromatography, and became clearly evident only upon purification by gas chromatography.

Crystallization procedures must also be used with care. Wright was unable to trap radioactivity in crystalline presumably all-trans retinoic acid after the administration of C^{14}-retinyl acetate to rats, or after its incubation with pig adrenal homogenates (45). In the present study crystallization of fraction II of bile derived from retinal treated animals with non-radioactive all-trans retinoic acid indicated

that about 10% of this fraction was free all-*trans* retinoic acid. But by gas chromatographic analysis of the remaining radioactivity in fraction II of bile, at least 40% more of fraction II was found to be *cis* isomers of retinoate. Thus great care must be used in interpreting crystallization data, since the radioactivity may largely reside in isomers other than the one specifically measured. In the present study retinoic acid has been unambiguously established as an intermediate in retinol metabolism *in vivo*. Whether it is an obligatory intermediate, or is only interconvertible with an unknown obligatory intermediate, has not yet been established.

In early studies retinoic acid could not be detected in tissues even after the administration of large doses of retinoic acid (39, 41-43). However, Jurkowitz, who devised a new acidic extraction method, did find free retinoic acid in human plasma after the administration of 100-120 mg of the acid. He justifiably criticized the extraction procedure used by other investigators (44). Using Jurkowitz's procedure, Krishnamurthy (37) also found free retinoic acid in chicks after the administration of large doses of the free acid. In the rat, however, free retinoic acid still could not be found. Using thin layer chromatography, Yagishita *et al.* were unable to detect free retinoate in extracts of rat liver or intestine as little as 5 minutes after the administration of 2 to 3 mg of retinoic acid (41). Similar results were reported by Zile and DeLuca (43). In their procedure a liver extract obtained from rats dosed with 1.5 mg of retinoic acid was passed through a series of organic extractions and finally purified by silicic acid and thin layer chromatography.

I was also unable to identify retinoic acid unambiguously in tissue extracts by thin layer chromatography. By use of a combination of ion exchange and gas chromatography, however, its presence in liver, bile, and intestine was clearly evident up to 4.5 hours after the administration of 4 mg of retinoic acid to bile duct cannulated rats. Since the amount of free acid in tissues is small, the inability of others to detect it may be attributed to its instability to light, heat and acid, poor extractability, and the presence of large quantities of lipid contaminants which obscured its spectrum and made its isolation difficult.

Although small amounts of retinoic acid persist in tissues, the major portion of an injected dose must be rapidly metabolized. A number of products of retinoate, several of which are water soluble, have been reported (24, 37, 41, 43, 46-50). Most of these metabolites have been poorly characterized, and indeed may be chemical artifacts of isolation instead of biological products. However, two groups of investigators have studied products of retinoic acid metabolism in sufficient detail to make worthwhile a comparison of their compounds with the compounds isolated in this investigation.

Zile and DeLuca (43) found a biologically active, radioactive product of retinoic acid in liver after fractionation of a lipid extract by silicic acid chromatography. The biologically active product was slightly less polar than retinoic acid and may well be similar to my fraction I, which had similar chromatographic properties on a thin layer plate of Silica Gel G. Smaller amounts of more polar but biologically inactive metabolites which were noticed, were not further characterized. Yagashita *et al.* (41) also reported the presence of a biologically

active product of retinoic acid in the liver and the intestine in addition to a non-polar, non-biologically active product. From its behavior on thin layer chromatography, their biologically active product seems similar to my fraction III.

The ultraviolet maxima of the compounds isolated by these two groups were well below 300 mμ, however, in sharp contrast to the maxima of 345 to 356 mμ found in all the major radioactive components isolated in this study. On the basis of spectra, these groups suggested that the metabolism of retinoate involves a shortening of the conjugated double bond system and perhaps cleavage of the side chain (41, 43). Present results do not support this view. Indeed the majority of the radioactivity in bile after the administration of retinoic acid was present in free or conjugated retinoic acid, and not in some oxidized metabolite. Since all of the radioactivity was not found in retinoate in our study, the possibility exists that a minor portion of retinoic acid may be reduced, hydroxylated, or cleaved. In all likelihood, however, the spectra observed by Yagishita (41) and Zile (43) were caused by ultraviolet absorbing contaminants which obscured the spectrum of retinoic acid. These contaminants were also found in all of the fractions isolated in the present study; some even co-crystallized with retinoic acid, and indeed were only eliminated after methylation and purification by gas liquid chromatography.

The distribution of radioactivity in ion exchange fractions of tissues is similar with retinal and retinol, but markedly different with retinoic acid. These patterns are explicable in terms of the known irreversibility of retinal oxidation to retinoate. On the other hand, the pattern of radioactivity in anion exchange fractions of bile

is approximately the same when any of the three substrates is injected. In addition, the relative amount and pattern of radioactivity excreted in the bile was the same whether large amounts (3 mg) or small amounts (10-25 μg) of retinoate were injected into the portal vein or into intestinal loops in vivo (50). Apparently the formation in liver of a water soluble metabolite, which is the major radioactive component of bile and was identified as a conjugate of retinoic acid, is a predominant pathway for the metabolism of various forms of vitamin A, once they have been converted to retinoic, or to a compound of the same oxidation-reduction state. Indeed the oxidation of retinal to retinoic acid seems to be the rate limiting step in this sequence.

The high excretory rate of retinol derivatives in the bile, which amounted to 20 to 60% of the injected dose in 24 hours, should not necessarily be interpreted as elimination of an undesirable, inactivated substance. In the intact animal Zachman (50) has shown that the radioactivity excreted in the bile is largely reabsorbed by the gut and re-excreted into the bile. Thus, these metabolites are involved in an enterohepatic circulation similar to that of the conjugated bile salts. Unlike retinol ester, retinoic acid may be "stored" in the form of a circulating water soluble entity.

Two bits of evidence suggest that some biologically active form of retinoic acid, although not necessarily the free acid itself, remains in tissues in appreciable amounts for rather extensive periods. Retinoic acid is toxic to rats when administered in daily doses of 2 mg per 100 g of body weight (76). Symptoms of toxicity only appear with relatively high doses of vitamin A derivatives. In the case of retinol, massive amounts of retinol ester are deposited in the liver and other organs. With retinoic acid, however, little free acid is found in

tissues within hours after injection. Since retinoic acid is more toxic than retinol (76), some form of the vitamin must persist in tissues to produce these symptoms. In regard to growth response, Malathi et al. (15) have shown that a single 500 μg dose of retinoic acid promotes the growth of vitamin A deficient rats for a period of 4 weeks, while a similar smount of retinol sustains growth for 5 weeks. Since retinoic acid is almost quantitatively converted to a water soluble conjugate within 48 hours, it is unlikely that the free acid produces these effects. It is attractive to propose that the retinoic acid conjugate or some product of it, which is produced rapidly and in large amounts in the liver, is reabsorbed by the intestine, and is readily soluble in plasma, is the biologically active entity responsible for both growth and toxic responses.

The retinoic acid conjugate of fraction III possesses an acidic function and one or more hydroxyl groups, properties which are consistant with only one conjugate commonly found in mammalian tissues, namely the glucuronides (77). Glucuronides are formed primarily in the liver and are excreted in the bile and urine (78, 79). Since β-glucuronidase has been found in almost every tissue and body fluid (80), a conjugate of glucuronic acid and retinoic acid would most likely be biologically active, or would become biologically active after enzymatic hydrolysis. The structure of the conjugate has not been unambiguously determined, however, nor has retinoyl glucuronide been synthesized chemically. Hence, the possibility cannot be overlooked that the polar moiety of the retinoate conjugate of fraction III may yet be unknown.

Although the all-*trans* isomer of retinol derivatives are more active than the *cis* forms in biological tests, the only well defined function of retinol, the visual cycle, requires the 11-*cis* isomer of retinal (20). The low activity of the *cis* isomers, including the 11-*cis* form, when orally administered in growth tests, has been attributed to an obligatory transformation to the all-*trans* forms, presumably in the liver and intestine, prior to its transport to the eye and other tissues (20-22). In the present investigation, however, *cis* isomers were shown to be incorporated directly into conjugates of retinoic acid found in the bile without previous equilibration with the all-*trans* form. Hence, the condensation reactions to form conjugates is apparently not specific for the all-*trans* form. Whether these *cis* retinoyl conjugates are active in biological systems *per se*, after isomerization to the all-*trans* form, or not at all, is presently unknown.

SUMMARY

1. Anhydro retinol, methyl retinyl ether, retinal, and methyl retinoate were separated by gas chromatography on short columns of silanized Gas Chrom P coated with 1% SE-30. The operating temperature was 150° C and the flow rate of carrier gas was 150 ml/minute. Retinol and retinyl acetate, which were dehydrated when chromatographed in this manner, were successfully separated after the column was treated with β-carotene and the flow rate was increased 6 fold. Methyl retinoate was isolated from tissue extracts on a longer, 4 fold more efficient column which contained 3% SE-30 and was operated at 180° with a flow rate of 50 ml/minute.

2. Ionic metabolites of retinol, retinal, and retinoic acid were separated from non-ionic metabolites and from massive amounts of non-ionic lipids by means of a gradient elution, anion exchange chromatographic system. Compounds in tissue extracts, when eluted with increasing concentrations of acetic acid in methanol, were separated reproducibly into three major fractions on columns of Bio-Rad AG2X8 in yields of 80-100%.

3. After the administration of 3 mg of C^{14}-retinal to bile duct cannulated rats, free radioactive retinoic acid was recovered from the bile. Thus free retinoic acid has been established as an intermediate in retinol metabolism, although not necessarily an obligatory intermediate.

4. The presence of small amounts of radioactive, free retinoic acid was unambiguously demonstrated in liver, the small intestine, and bile up to 6 hours after the administration of 2-4 mg of C^{14}-retinoic acid to bile duct cannulated rats.

5. Non-acidic conjugates (fraction I) were found in the tissues after the administration of retinol, retinal, or retinoic acid. After the administration of retinol or retinal, retinol ester appeared in the liver and the intestine but not in the bile. After retinoate administration, an ester of retinoic acid was found in the liver and in the bile.

6. Acidic polar conjugates (fraction III) were found in the bile after the administration of radioactive retinol or retinal, and in the liver and intestine as well as the bile after the administration of radioactive retinoic acid. An acidic conjugate of retinoic acid accounted for 23% of fraction III of bile after administration of retinal and >50% of fraction III of bile after retinoate administration. This conjugate was water soluble, polar, possessed a carboxyl group or other acidic function, and contained one or more hydroxyl groups.

7. After the administration of C^{14}-retinoic acid to bile duct cannulated rats, the major recovered components were free retinoic acid and its conjugates, rather than further oxidized or partially degraded metabolites.

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VITA

Percy Elford Dunagin, Jr. was born August 29, 1934, at Spartanburg, South Carolina, where he received most of his elementary and high school education. After spending his freshman year at Southern Missionary College in Tennessee during 1952-53, he served in the Army Security Agency of the United States Army from January, 1954 until January, 1957. He then resumed his undergraduate education at Southern Missionary College, where he received the degree of Bachelor of Arts with honors in 1960. During the summer of 1960 he served as an instructor in chemistry at Southern Missionary College. He enrolled in the Graduate School of the University of Florida in September, 1960, worked in the Department of Biochemistry as a graduate assistant until 1961, and remained as a predoctoral trainee in that department until 1964. From 1960 until the present time, he has pursued his work toward the degree of Doctor of Philosophy.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Medicine and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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